The Role of Caspase Inhibitors in Protecting the Myocardium from Ischemia Reperfusion Injury

al-Rajaibi, Hajar Mohammed Amor

Award date:
2008

Awarding institution:
Coventry University

The Role of Caspase Inhibitors in Protecting the Myocardium from Ischemia Reperfusion Injury

Hajar M. AL-Rajaibi

BSc, MD, MSc

A thesis submitted in partial fulfilment of the requirements
for the degree of Doctor of Philisophy.

School of Health and Life Sciences

Coventry University

2008

Abstract

Introduction

Rapid restoration of blood flow to ischemic myocardium is essential, however it causes further injury called reperfusion injury. Apoptosis contributes significantly to cardiomyocyte cell death during ischemia reperfusion injury, in which caspase family proteases play an essential role as they are the executioners of apoptosis. Caspase inhibitors showed promising cardioprotective results when administered before ischemia or at the start of reperfusion. However, before applying them in pre clinical studies of myocardial ischemia, several investigations needed to be taken to determine their therapeutic window post reperfusion, their effect on functional recovery of myocardium post ischemia, their mechanism of action.

Methods

Isolated perfused rat hearts were subjected to 35 min ischemia followed by 2 hr reperfusion where caspase inhibitors [broad spectrum caspase inhibitor (ZVAD, 0.1μM), specific caspase 3 inhibitor (DEVD, 0.07μM)] were added at the start of reperfusion, 15, 30 and 60 min after starting reperfusion at the presence or absence of Wortmannin (WORT, 100nM, PI3-kinase inhibitor). Hearts underwent triphenyl tetrazolium staining for infarct size assessment, or were frozen for Western blot analysis. Freshly isolated adult rat ventricular myocytes were subjected to 6 hr hypoxia followed by either 18 hr, where caspase inhibitors (ZVAD, 25μM and DEVD, 25μM)] were added at the start of reoxygenation, 15, 30 and 60 min after starting reoxygenation at the presence or absence of Wortmannin (WORT, 100nM). Cardiomyocytes were analysed for viability, apoptosis, necrosis and intracellular caspase-3 activity using flow cytometry analysis. Isolated adult rat ventricular papillary muscles were subjected to 35 min hypoxia followed by 100 min reperfusion where caspase inhibitors [ZVAD (0.1 μM, 2.5μM) and DEVD (2.5μM)] were added at the start of reperfusion throughout. Power output was measured using work loop technique.

Results

Both caspase inhibitors significantly reduced infarcted tissue within the risk zone, apoptotic, necrotic cellular death and intracellular caspase 3 activity level when administered at the start of reperfusion, 15, 30 and 60 min after initiation of reperfusion. Work loop studies showed that the broad spectrum caspase inhibitors at high dose of 2.5μM significantly improved post ischemic power out put recovery, however the specific caspase 3 inhibitor has no significant effect on power output recovery post ischemia. Inhibition of PI3-Akt by WORT at the start of reperfusion abrogated the cardioprotective effect of both caspase inhibitors. However, WORT did not block the cardiprotection of both caspase inhibitors when administered at 15 min and 30 min after starting reperfusion. Western blot analysis showed that cytochrome c is released to cytosol during ischemia/reperfusion injury; however both caspase inhibitors failed to inhibit cytochrome c release during reperfusion injury at all time points during reperfusion. Western blot showed a significant increase in phospho-Akt at 5 min and 10 min reperfusion in both caspase inhibitors treated groups compared to ischemic control group. However there was no significant increase at 20 min and 120 min reperfusion when compared to ischemic control. WORT blocked the increase in phospho-Akt at 10 min reperfusion.

Conclusion

This study is the first to show that administration of caspase inhibitors at various time points post reperfusion can still reduce myocardial injury, which implies that the therapeutic window of caspase inhibitors in myocardial infarction treatment can be extended for an hour after starting reperfusion. It also showed that broad inhibition of caspase resulted in a significant functional recovery post ischemia using for the first time work loop technique to measure the power output. This study is also the first to show that cardioprotection by caspase inhibitors at early reperfusion phase is mediated via PI3-Akt cell survival pathway.

Acknowledgements

I gratefully acknowledge my supervisor Dr. Helen Maddock for all her advice, support and great supervision throughout my PhD study. Thank you for all your valuable advice, kindness, patience and encouragement. I would like also to thank my second supervisor Dr. Rob James for all his help, advice and encouragement throughout my research. Thank you for your valuable contribution to my PhD study.

I would like to thank without a doubt my friend Afthab, who was like a brother for me in UK. His support and encouragement is highly appreciated. I'm also grateful to my friends Almas, Pushba, Patricia and Islam for the lovely times we spent together in the lab.

I would also like to thank the laboratory technicians Mark, Adrian, Sue and Angela for all the help, patience and advice they provided.

I would like to acknowledge Sultan Qaboose University, for awarding me this full time scholarship to do my PhD.

Finally, my deepest appreciation without doubt goes to my family and friends, to whom I dedicate this work, for all the encouragement throughout my work.

Table of Contents

Table of Figures

List of of Tables

List of Abbreviations

2-Cl-IBMECA	2-chloro-N(6)-(3-iodobenzyl)-5'-N-methylcarbamoyladenosine
AIF	Apoptosis inducing factor
ANOVA	Analysis of variance
ANT	Adenine nucleotide translocator
Apaf-1	Apoptotic protease activating factor-1
ATP	Adenosine Tri Phosphate
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CAD	Caspase activated Dnase
Caspase	Cysteine aspartate specific proteases
CF	Coronary flow
Ac-DEVD-CMK	Ac-Asp-Glu-Val-Asp-CH2Cl cell permeable and irreversible inhibitor of caspase-3
DD	Death domain
DIABLO	Direct IAP-binding protein with low pI
DNA	deoxyribonucleic acid
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
ERK	Extracellular signal regulated kinase
FADD	Fas associated death domain
HR	Heart rate
HRP	Horseradish peroxidase
I/R %	Infarct size to risk area ratio
FasL	Fas ligand
FADD	Fas ligand associated death domain
GSH	Glutathione

JNK	c-jun N-terminal kinase
KATP	ATP potassium sensitive channel
KHB	Krebs Henseleit Buffer
LVDP	Left ventricular developed pressure
MAPK	Mitogen activated protein kinase
MPTP	Mitochondrial permeability transition pore
OAA	Oxaloacitic acid
PARP	Poly (ADP ribose) polymerase
PMNs	Polymorphonuclear leucocytes
PS	Phosphotidylserine
PVDF	Polyvinyl Difuoride
RISK	Reperfusion injury salvage kinases
SEM	Standard error of the mean
SMAC	Second mitochondria-derived activator of caspase
TCA	Tricarboxylic acid cycle
TNB	5-thio-2-nitrobenzoic acid
TNF	Tumor necrosis factor
TBS	Tris buffer saline
TRADD	TNF-rceptor associated death domain
TRAIL	TNF-related apoptosis inducing ligand
TTC	Triphenyltetrazolium chloride
VDAC	voltage-dependent anion channel
WORT	Wortmannin
ZVAD	Z-Val-Ala-Asp(OMe)-CH2F cell permeable irreversible inhibitor of caspase-1 like proteases

CHAPTER 1

Literature Review

1.0 Introduction

Acute myocardial infarction or commonly known as “Heart attack” is the leading cause of death in developed countries such as UK and USA, but third to AIDS and lower respiratory infections in developing countries (Okrainec *et al*., 2004). The estimated incidence rate of myocardial infarction in UK is about 55,000 heart attacks per year in men aged under 75 and 20,000 in women giving a total of 75,000 (Allender *et al*., 2007).

Coronary arteries are the blood vessels that supply the heart muscle with blood and oxygen. Blockage of a coronary artery deprives the heart muscle of blood and oxygen, causing injury and subsequently death to the heart muscle (Myocardial infarction). Atherosclerosis is the common cause of myocardial infarction (Ross, 1999). It involves the formation of fatty deposits (plaques) on the walls of the coronary arteries. Rupture of the atherosclerotic plaques causing a blood clot to be formed on the surface of the plaque, which can cause a complete block of the coronary artery causing a heart attack (Ross, 1999). The cause of rupture that leads to the formation of a clot is largely unknown, but contributing risk factors may include cigarette smoking or other nicotine exposure, elevated blood cholesterol, elevated levels of blood catecholamines (i.e adrenaline), high blood pressure, and other mechanical and biochemical forces (Altman, 2003).

Acute myocardial infarction treatment is considered a challenge for clinicians and researchers because of its associated high rate of mortality and morbidity. In UK, around one in five men and one in six women die from the disease. Coronary heart disease caused just under 114,000 deaths in the UK in 2003 (Allender *et al.*, 2007). Recent statistics published by the American Heart Association (AHA) indicate that approximately 25% of men and 38% of women will die within 1 year of having an initial recognized myocardial infarction, and people who survive the acute stage have an increased risk of illness and death that is up to 15 times higher than that of the general population (Thom *et al.*, 2006).

A lot of interventional programs attacking the primary risk factors have been developed, however their impact is still limited (Yellon & Baxter, 2000). Therefore, there is a need for effective forms of secondary prevention and treatments that will be able to preserve myocardial viability during acute myocardial ischemia insult. To date the only proven way of salvaging ischemic heart tissue is urgent revascularisation and restoration of blood flow (de Boer *et al.*, 1994). Currently, the most effective methods used in standard clinical reperfusion treatment are thrombolysis, percutaneous coronary angioplasty (primary PCTA), or bypass surgery (de Boer *et al.*, 1994, Wong & White, 2003.). Although early reperfusion halts the ongoing process of ischemic injury, paradoxically the process of reperfusion can itself causes myocyte cell death, a phenomenon called reperfusion injury (Hearse & Bolli, 1992, Yellon & Baxter, 2000). This phenomenon may have major clinical implications because reperfusion after a period of ischemia occurs not only during myocardial infarction but also in other frequent and important clinical conditions such as

angina and cardioplegic arrest during cardiac surgery. Therefore pharmacological therapy administered during reperfusion is needed to limit ischemia/reperfusion injury.

1.1 Pathogenesis of ischemia/reperfusion injury

The inability of the myocardium to meet oxygen and nutrient supply with respect to demand is referred to as myocardial ischemia (Asano *et al.*, 2003). Numerous conditions may elicit an ischemic myocardium, the most important and frequent cause is atherosclerosis of coronary arteries. Atherosclerosis occurs as a result of the development of atherosclerotic plaques that protrude into the lumen of the coronary arteries resulting in obstruction of blood flow (Ross, 1999). A 50 % reduction in the coronary artery diameter can lead to a significant reduction in the blood flow and ischemia can occur (Hoffman *et al.*, 2004).

Reduced blood flow during ischemia indicates that there is a reduced flow of oxygen to the ischemic tissues. While there is reduced oxygen, there is a reduction in oxidative phosphorylation, which leads to a reduction in the rate of formation of ATP, which is the primary source of energy substrates for myocytes (Guyton, 1991). ATP levels decrease by 65% after 15 minutes of ischemia and by 90% after 40 minutes of ischemia (Reimer & Jennings, 1986). As a result, the muscle has less ability to generate active force, and ion homeostasis is lost. The ATP dependent ion exchangers and transporters can no longer function, with the result that potassium ions start to leak into the extracellular space, and calcium ions leak into the cell. This leads to electrical instability and failure of the cells to relax. This process of ischemia proceeds until the myocytes are swollen, acidotic, and show

signs of structural disorganization (Maxwell & Lip, 1997). In addition, during ischemia, glutathione (GSH) is also depleted. GSH is a sulphydryl that acts to protect the cellular proteins and lipids from oxidative damage. Therfore, reduced glutathione results in exacerbation of oxidative stress (Patterson & Rhoedes, 1988).

Cell death following myocardial ischemia is dependent on both the duration and the severity of myocardial ischemia (DeBoer *et al*., 1983). Therefore, reperfusion of ischemic myocardium carried out at the earliest possible time following coronary occlusion is considered as an important goal. Although reperfusion is essential and an absolute prerequisite for survival of the ischemic myocardium, the process itself can lead to additional myocardial injury and other associated pathologies which are referred to as "reperfusion injury" (Hearse & Bolli 1992, Yellon & Baxter, 2000).

Reperfusion injury was described in the literature as early as 1960 by Jennings *et al*. They reported both structural and electrophysiological changes associated with coronary reperfusion and they were the first to suggest that reperfusion accelerated the necrotic process of irreversibly injured myocytes. Since then, numerous studies described the same phenomenon and its underlining mechanisms and therapeutic approaches which have been reviewed by different papers (Cohen 1989, Forman *et al.* 1990, Hearse, 1990, Jennings *et al.*, 1995, Lucchesi, 1997, Frangogiannis *et al.* 1998, Jordan *et al.* 1999, Park & Lucchesi 1999). The major controversy about the nature of reperfusion injury is whether reperfusion merely exacerbates tissue damage that would in any case have occurred during ischemia as suggested by Jennings and his group (1960) or whether there is an additional injury caused

merely by reperfusion itself. Therefore, defining reperfusion injury is not straightforward, but it can be defined as "those metabolic, functional, and structural consequences of restoring coronary arterial flow that can be avoided or reversed by modification of the conditions of reperfusion" (Park & Lucchesi, 1999).

Reperfusion injury can be considered as a syndrome consisting of different clinical and pathological events including: reperfusion arrhythmias, vascular damage and no-reflow, myocardial functional stunning, and extension of the mass of tissue injury (Maxwell & Lip, 1997). Several mechanisms and mediators of reperfusion injury have been described. The most frequently cited mechanisms are oxygen free radicals, polymorphonuclear leucocytes, alteration in calcium handling and altered myocardial metabolism (Maxwell & Lip, 1997).

1.1.1 Oxygen free radicals

Oxygen free radicals or reactive oxygen species (ROS) play an important role in the pathophysiology of ischemia reperfusion injury. Oxygen free radicals have unpaired electron, which makes them extremely reactive and capable of inducing oxidative modification of other molecules. According to existing evidence, molecular oxygen, when reintroduced into a previously ischemic myocardium, undergoes a sequential reduction leading to the formation of oxygen free radicals within a few minutes of reperfusion (Zewier 1988, Bolli *et al.*, 1989). ROS can also be generated from sources other than reduction of molecular oxygen. These sources include enzymes, such as xanthine oxidase, cytochrome oxidase, and cyclooxygenase, and the oxidation of catecholamines (Singal *et*

al., 1983). In addition, reperfusion stimulates neutrophil activation and accumulation, which in turn are considered as potent stimuli for ROS accumulation (Jordan *et al.,* 1999).

The damage produced by ROS is through reaction with polyunsaturated fatty acids, resulting in the formation of lipid peroxides and hydroperoxides that damage the sarcolemma and impair the function of membrane-bound enzyme systems (Verma *et al.,* 2002). In addition, free radicals stimulate the endothelial release of platelet activating factor, which attracts more neutrophils and amplifies the production of oxidant radicals and the degree of reperfusion injury (Forde & Fitzgerald, 1997). Reactive oxygen species also reduce nitric oxide, thus exaggerating endothelial injury and microvascular dysfunction (Verma *et al.,* 2002). In addition, endogenous oxidant scavengers reduced during ischemia, which exacerbates the oxidative stress (Park & Lucchesi, 1999).

ROS are strongly implicated in the pathogenesis of postischemic myocardial stunning (Bolli *et al.,* 1988), necrosis (Tavazzi *et al.,* 1998) and programmed cell death (apoptosis) (Maulik *et al.,* 1998). The effects of antioxidants and free radical scavengers have been investigated in numerous experimental and clinical models with varying success (Przyklenk, 2001). This variability in outcomes can be attributed to species differences in collateral blood flow and the different durations of ischemic insults used in these experiments. Even though positive results were shown in classical animal models of experimental ischemia and reperfusion, human clinical studies with antioxidants have been disappointing as they have failed to show any beneficial effects after using human

recombinant superoxide dismutase (anti-oxidant) (Flaherty *et al.*, 1994). Conflicting results between animal studies and human trials may be the result of species differences.

Recently Kevin *et al.,* (2005) showed that ROS involved in the signalling cascade for cardiopotection induced by brief exposure to a volatile anaesthetic agents. ROS, therefore, although injurious in large quantities, can have a paradoxical protective effect within the heart in smaller quantities.

1.1.2 The polymorphonuclear leucocytes (PMNs)

Accumulating evidence has indicated that myocardial ischemia elicits an acute inflammatory response that is greatly augmented by reperfusion and that a significant part of total myocardial injury after ischemia and reperfusion is attributable to the inflammatory response (Park & Lucchesi, 1999). The polymorphonuclear leucocytes (PMNs) represent a major cellular component of that inflammatory response. The role of the PMNs in myocardial ischemia and reperfusion injury was first demonstrated by histological studies showing a direct correlation between both the duration of ischemia and infarct size and the extent of PMNs accumulation within the myocardial tissue (Romson *et al.,* 1983). In addition, induction of neutropenia (lack of PMNs), as well as the inhibition of PMNs adhesion (Simpson *et al.,* 1990), results in a cardioprotective effect, further implicating the importance of PMNs in contributing to the development of myocardial reperfusion injury.

PMNs have the capacity to produce oxygen-derived free radicals (Metha *et al.,* 1988) when activated by appropriate stimuli as mentioned earlier. In addition to the damage caused by

free radicals production, PMNs degranulate to release proteases, collagenases, lipoxygenases, phospholipases, elastases and myeloperoxidase. Elastase is a major contributor to PMNs mediated damage, due party to the effect of its highly cationic nature on membrane charge distribution. Elastase also hydrolyzes the extracellular matrix components elastin, fibronectin, and collagen types III and IV (Jordan *et al.,* 1999). In addition, PMNs activation stimulates phospholipase A2 and generates leukotriene B4 and platelet activating factor. The latter two are potent stimulants of PMNs chemotaxis, degranulation, and adhesion to endothelial cells, which might then amplify neutrophil recruitment and injury (Jordan *et al.,* 1999). Cytokine-induced PMNs activation increases not only adherence properties but also cytoskeletal rigidity, so the PMNs are no longer flexible, and so they then embolize (Jordan *et al*., 1999).

However, the exact role of PMNs in the pathogenesis of reperfusion injury is still a point of controversy. Some studies completely discredit the role of PMNs in reperfusion injury. Briaud and his group (2001) used mice with a deficiency (via gene deletions) of P-selectin and intercellular adhesion molecule-1 (ICAM-1), which mediate early interaction and adhesion of neutrophils to coronary endothelial cells and myocytes after myocardial ischemia and reperfusion. They found that the infarct size assessed 24 hours after reperfusion was not different between wild-type and deficient mice after 30 minutes and 1 hour of ischemia. In addition *in-vitro* models of ischemia/reperfusion which doesn't involve blood or any PMNs interactions in their setting (e.g. Langendorff model and isolated cell model, which we used in our studies), demonstrated occurrence of reperfusion injury after ischemia.

1.1.3 Alteration in calcium homeostasis

Changes in intracellular calcium homeostasis play an important role in the development of reperfusion injury. Cytosolic calcium concentration is increased during prolonged ischemia (Marban *et al.*, 1990) and this is often exacerbated following reperfusion (Nayler *et al.*, 1980). This reperfusion induced calcium overload has been explained by Grinwald (1982) as follows: during ischemia, intracellular sodium accumulates due to energy depletion, but Na^+–H^+ exchange is inhibited by the concomitant acidosis. Upon reperfusion, the rapid reversal of acidosis reactivates Na^+-Ca^{2+} exchange at a time when sodium overload has not yet been resolved, driving calcium into the cells. Mitochondrial matrix calcium overload considered one of the key factors responsible for mitochondrial permeability transition pore (MPTP) opening during reperfusion injury (Halestrap *et al*., 2004). MPTP opening results in release of pro-apoptotic proteins like cytochrome c and AIF (Halestrap *et al*., 2004) thus, initiating apoptosis, which will be discussed later. In addition to intracellular calcium overload, alterations in myofilament sensitivity to calcium have been implicated in reperfusion injury (Verma *et al.*, 2002). Activation of calcium-dependent proteases (calpains) with resultant myofibril proteolysis has been implicated too. These “calpains” are enzymes that cleave other proteins when cell calcium concentration is elevated (Gao *et al.*, 1996, 1997).

1.1.4 Altered myocardial metabolism

Reperfusion of an ischemic myocardium results in altered metabolism, which may contribute to delayed functional recovery (Verma *et al.*, 2002). Lack of oxygen during ischemia induces anaerobic metabolism, with a net production of lactate, which persists

during reperfusion (Rao *et al.,* 2001). In addition, the activity of mitochondrial pyruvate dehydrogenase (PDH) is inhibited by 40% after ischemia and remains depressed up to 30 minutes after reperfusion (Merante *et al.*, 1988). Therefore, the persistent anaerobic lactate production during reperfusion contributes significantly to impaired ventricular function recovery (Rao *et al.,* 2001). These findings suggest that the persistent anaerobic metabolism may be an important contributor to reperfusion injury, therefore improving the recovery of aerobic myocardial metabolism may serve as an important target to attenuate reperfusion injury. Different studies that used interventions to improve aerobic metabolism using insulin or adenosine showed rapid recovery of aerobic metabolism and left ventricular function after post-cardiac surgery reperfusion (Rao *et al.*, 2000).

1.2 Cell death during Ischemia/Reperfusion injury

Necrosis is believed to be the major form of pathological cell death after ischemia/reperfusion injury. However, recent evidence suggests that apoptosis (programmed cell death) is a significant contributor to cell death during ischemia/reperfusion injury (Saraste *et al*., 1997, Zhao *et al*., 2000, Gottlieb *et al*., 1994).

Necrosis and apoptosis differ in several morphological and cellular regulatory features (Kerr *et al.,* 1972). Necrosis is a destructive irreversible death process (Searle *et al.,* 1982). It involves swelling of the cell and its organelles, disruption of mitochondria, membrane rupture and cell lyses and significant inflammatory response (Searle *et al.,* 1982). In contrast apoptosis is energy dependent and a genetically controlled programmed cell death (Kerr *et al.,* 1972). It is originally defined by its morphological characteristics, which

include cell shrinkage, chromatin condensation, DNA fragmentation, formation of cytoplasmic blebs and apoptotic bodies, no loss of cell integrity and no inflammatory response (Kerr *et al.,* 1972). This form of cell death requires caspase activation, as these enzymes can activate the endonucleases responsible for DNA degradation (Smulson *et al.,* 2000).

A large body of publications have shown that apoptosis is induced during ischemia/reperfusion injury (Gottleib *et al.,* 1994, Fliss *et al.,* 1996, Kajstura *et al.,* 1996, Freud *et al.,* 2000, Zhao *et al*., 2000, Borutaite *et al.,* 2003); however whether apoptosis is induced during the ischemia period or after starting reperfusion is still controversial. One of the earliest studies in apoptosis showed that apoptosis was detected in ischemic myocardium after 30 min of ischemia and 4 hour of reperfusion in the rabbit myocardium, but not in ischemic only myocardium (Gottleib *et al.,* 1994). The same observation was confirmed by Zhao *et al.,* (2000), where they showed that apoptosis only appeared in dog myocardium subjected to a brief period of ischemia followed by reperfusion (60 min ischemia and 6 hrs reperfusion), and not in ischemic tissue (7 hrs ischemia) without reperfusion. Freud *et al.,* (2000) also showed that in the experiments with 90 min ischemia in isolated canine hearts, apoptotic cell death was only observed after initiation of reperfusion. In addition Dumont *et al.,* (2000) showed that the level of apoptosis is dependent on reperfusion duration. Studies on isolated myocardial rat cells have again shown that apoptosis is the predominant mode of cell death during reoxygenation, but that non-apoptotic cell death predominates during prolonged hypoxia alone (Kang *et al.,* 2000). In contrast, Fliss *et al.,* (1996) showed that either continuous ischemia or ischemia followed

by reperfusion can cause apoptosis in the rat model of coronary artery occlusion. Anvesa *et al.,* (1998) quantified the level of apoptosis and reported that, the level is 30 times higher after 2 hours of ongoing ischemia when compared with the start of ischemia. In addition Borutaite *et al.,* (2003) showed that activation of caspase, which are the hallmark of apoptosis, was dependent on the time of ischemia. The reasons for these contradictory results are unclear, but may be related to species differences, degree of anoxia used and different apoptosis assays used.

In general, the above studies may suggest that apoptosis is either triggered or accelerated during reperfusion. The fact that apoptosis is an energy dependent process and ATP levels are depleted during ischemia and replenished on reperfusion may explain why apoptosis might be triggered and accelerated with reperfusion (Leist *et al.,* 1997, Freud *et al.,* 2000). This raises the importance of adjunctive therapy introduced during reperfusion to limit apoptosis and consequently protect myocardium from further injury.

Even though human studies are limited, several reports have documented the occurrence of apoptosis in human heart specimens obtained from patients who died following myocardial infarction (Olivetti *et al.,* 1996, Saraste *et al.,* 1997). In addition autopsies from patients with acute myocardial infarction suggest that apoptosis is the early and predominant form of cell death in infarcted human myocardium, and its appearance is accelerated in reperfused myocardium (Veinot *et al.,* 1997). The apoptotic cells were significantly more numerous in the border zone of the infarcted tissue (Olivetti *et al.,* 1996, Saraste *et al.,* 1997). This distribution suggests that apoptosis may be in part responsible for extending

infarction over time after the onset of reperfusion (Olivetti *et al.,* 1996). Zhao *et al*., (2001) found that myocardial apoptosis in the peri-necrotic myocardium progressively developed during the reperfusion period and was consistent with extension of infarction size and attenuation of contractile function. Later Zhao *et al.,* (2003) found that inhibition of apoptosis during reperfusion was associated with a reduction in infarction size and improvement in contractile function. These two studies suggested that apoptosis may exacerbate myocardial injury. Therefore, these studies highlighted the importance of reducing apoptosis which will limit infarction extension and preserve myocardial function.

Even though the above studies showed the significance of apoptosis, the relationship between apoptotic and necrotic cell death during ischemia reperfusion injury is another unresolved problem in the literature. It has been shown that these two forms of cell death occur simultaneously during the reperfusion phase (Zhao *et al.,* 2001). The anti-apoptotic proteins Bcl-2 and Bcl-xL effectively retarded the chemical hypoxia-induced necrotic cell death (Shimizu *et al.,* 1996). The necrotic cell death is also retarded by inhibitors of ICE (-like) proteases, including interleukin-1beta converting enzyme (ICE), which are common mediators of apoptosis (Shimizu *et al.,* 1996). In addition pharmacological inhibition of apoptosis using caspase inhibitors (apoptosis excutioners) resulted in attenuation of both apoptotic and necrotic cells (Yoita *et al.,* 1998, Mocanu *et al.,* 2000, Zhao *et al.,* 2003). These results suggest the possibility of cross-talk between the two forms of cell death and they may share common molecular pathways (Shimizu *et al.,* 1996). Therefore Gottlieb & Engler (1999) reported that the the distinction between apoptosis and necrosis is blurred in

the case of a pathologic insult such as ischemia-reperfusion injury and suggested that it is more useful to view cell death in the context of whether or not it can be prevented.

1.3 Caspase (the executioners of apoptosis)

Caspase (**C**ystine **asp**artate specific Protein**ase**) represent the downstream effectors and the executionary machine of apoptosis (Cryns & Yaun, 1998). The idea that caspase activation is necessary and sufficient for apoptotic cell death is based on the observation that the caspase product of the CED-3 gene is essential for all the developmental programmed cell deaths in the nematode worm *Caenorhabditis elegans* (Thomberry *et al.*, 1992). Yaun *et al.*, (1993) showed a homology between CED-3 product and interleukin-1β-converting enzymes (ICE, Caspase-1). More recently researchers have identified other caspase of the large family of mammalian CED-3/ICE-like proteases (Thornberry & Lazebnik, 1998). Currently there are about 14 known caspase (Nicholson, 1999). They exist within the cell as inactive pro-forms or zymogens. These zymogens can be cleaved to form active enzymes following the induction of apoptosis (Cryns & Yaun, 1998).

Caspase constitute a family of cysteine proteases that cleave their substrates after aspartic acid residue (Nicholson, 1999). The inactive caspase (zymogenes) composed of a large and a small subunit preceded by a variable length N-terminal prodomain (Nicholson, 1999). Both the large and the small subunits, which together make up the active form of the enzyme, are liberated from the zymogen through proteolysis at specific Asp residues residing between the prodomain, long and short subunits. This proteolysis results in activation of caspase via exposure of the active sites. Crystallographic studies showed that

the active caspase is a tetramer of two heterodimers, thus containing two active sites (Nicholson, 1999). Active caspase recognize a very short tetrapeptide (P1,P2,P3,P4) sequence within targeted substrate polypeptides and these motifs have formed the basis for substrate specificity as well as specific peptide inhibitors for caspase (Nicholson, 1999).

The caspase implicated in apoptosis can be divided into two functional subgroups based on their known or hypothetical roles in the apoptotic process: initiator caspase (caspase-2, -8, -9, and -10) and effector caspase (caspase-3, -6, and -7) (Enari *et al.,* 1996). Usually, initiator caspase, once activated, will activate the downstream effector caspase in a cascade-like pattern (Slee *et al.,* 1999). The effector caspase are responsible for the cleavage of the key cellular proteins that leads to the typical morphological changes observed in cells undergoing apoptosis (Fan *et al.,* 1999).

1.4 Caspase activation pathways

There are a number of pathways through which caspase can be activated in cells and eventually commit cells to apoptosis. The well studied pathways in cardiomyocytes involve death receptor pathway (extrinsic pathway) and the mitochondrial pathway (intrinsic pathway) (Gill *et al.,* 2002).

1.4.1 Death receptor pathway (extrinsic pathway)

Death receptors are cell surface receptors that transmit apoptosis signals initiated by specific ligands (Ashkenazi & Dixit, 1998). These receptors include tumour necrosis factor receptor 1 (TNFR1, also called p55 or CD120a), Fas (CD95 or Apo1), death receptor 3

(DR3), death receptor 4 (DR4) and death receptor 5 (DR5). The binding of TNFα, FasL (FAS-Ligand) and TRAIL (TNF-related apoptosis-inducing ligand) to their receptive receptors initiates signal transduction pathways that result in the induction of apoptosis (Muzio *et al.,* 1996). These death receptors contain death domain (DD) in their cytoplasmic portion (Chinnaiyan *et al.,* 1995). The death receptors interact via their DD with intracellular DD- containing adapters, such as FADD (Fas-associating protein with DD) and TRADD (TNF receptor associated DD) and recruit these adapters to the cell membrane (Chinnaiyan *et al.,* 1995, Hsu *et al.,* 1999), Figure 1.1. Thus, binding of Fas ligand to the Fas receptor leads to clustering of the Fas receptor's DD. The adapter, FADD, binds through its own DD to the clustered receptor DD. FADD also contains a 'death effector domain' (DED) that binds to an analogous domain within procaspase-8 protein. Upon recruitment by FADD, procaspase-8 oligmerisation drives its own activation to caspase 8 through self cleavage (Yue *et al.,* 1999), Figure 1.1. Caspase 8 can activate other effector caspase either directly or through activating the mitochondrion-mediated pathway by truncating Bid (a pro-apoptotic Bcl-2 family member), a kind of proapoptotic protein in the cytosol, into its active form, tBid. tBid will trigger the activation of the mitochondrion pathway and apoptosis is induced (Li *et al.,* 1998), Figure 1.1. The activation pathway mediated by procaspase-10, with a DED-containing prodomain, is similar to that mediated by procaspase-8. Caspase-10 functions mainly in the apoptosis of lymphoid cells (Wang *et al.,* 2001).

The involvement of the receptor death pathway in cardiomyocyte death during ischemia reperfusion injury is supported by studies that report expression of Fas and Fas ligand in the

heart (Tanaka *et al.,* 1994, Yamaguchi *et al.,* 1999). Furthermore, enhanced expression of Fas found in association with increased apoptosis in experimental models of myocardial infarction (Kajstura *et al.,* 1996) and hypoxia (Tanaka *et al.,* 1994). Similarly, TNF was found to be elevated in serum of humans with heart failure (Levine *et al.,* 1990), where apoptosis played a significant role (Gill *et al.,* 2002). TNF receptors were also shown to be expressed by cardiomyocytes (Krown *et al.,* 1995). However, the role of the death receptor pathway in cardiac myocyte apoptosis during ischemia reperfusion injury has been controversial. There were some studies demonstrated data suggesting that the CD95/Fas is directly involved in cell death after myocardial ischemia using isolated rat heart model and primary adult rat cardiomyocytes culture model (Jeremias *et al*., 2000) and *in vivo* model (Lee et al., 2003). However, Comiz *et al*., (2005) reported that mitochondrial permeability transition rather than activation of Fas plays a pivotal role in cardiomyocyte death after a prolonged ischemia-reperfusion insult in mice model. Moreover, mice treated systemically with an activating Fas antibody die from massive hepatocyte apoptosis but exhibit no cardiac pathology (Ogasawara *et al*., 1993). These studies raise the possibility that Fas activation may not be enough to induce apoptosis efficiently in cardiac myocytes.

Figure 1.4: Apoptotic death receptor pathways (Fan *et al*., 2005).

1.4.2 Mitochondrial pathway (intrinsic pathway)

The intrinsic death pathway is regulated by mitochondrial activity. Mitochondria play an important role in the regulation of apoptosis. Not only do they provide the energy essential for completion of apoptosis, but they also release important proapoptotic factors into the cytosol (Liu *et al.,* 1996, Zamzami *et al.,* 1997). Cytochrome c and AIF (apoptosis-inducing factor) are released from the mitochondrial intermembrane space in response to cell stimulation (Figure 1.2) (Liu *et al.,* 1996). Then cytochrome c directly forms a trimetric complex with Apaf-1 in an ATP dependent manner. This complex activates procaspase-9 resulting in cleavage into caspase 9, which then processes and activates other caspase to orchestrate the biochemical execution of cells (Li *et al.,* 1997). The activated caspase-3 will then activate pro-caspase-9 and form a positive feedback activation pathway (Fan *et al.,* 2005) (Figure 1.2). AIF exerts its action directly by stimulating caspase (Zhao *et al.,* 2002). Recently a new mitochondrial factor was found to induce apoptosis called second mitochondria-derived activator of caspase (SMAC; Due e*t al.,* 2000) also known as DIABLO (direct IAP-binding protein with low pI) (Verhagn *et al.,* 2000). Upon receiving

pro-apoptotic signals, SMAC/DIABLO is released from mitochondria into the cytosol where it interacts with IAPs (Inhibitory protein of apoptosis) and abrogates their caspase inhibitory activity, thereby potentiating apoptosis.

The involvement of the mitochondrial-cytochrome c mediated pathway in cardiomyocytes death during ischemia reperfusion injury is addressed by Bialik *et al.,* (1999). They showed that deprivation of serum and glucose, components of ischemia *in vivo,* resulted in myocyte apoptosis (as determined by nuclear fragmentation), internucleosomal cleavage of DNA, and processing of caspase substrates. Furthermore, it has been reported that mitochondrial cytochrome *c* release, activation of caspase-3, and PARP cleavage are involved in reactive oxygen species-induced cardiomyocyte apoptosis, a situation occurring during ischemia reperfusion injury (Von Harsdorf *et al.,* 1999). Kang *et al.,* (2000) showed that during reoxygenation of adult rat ventricular myocytes, cell death occurred predominantly via apoptosis associated with appearance of cytosolic cytochrome c and activation of caspase-3 and -9. These results suggest that apoptosis predominates in cardiomyocytes after reoxygenation through a mitochondrial-dependent apoptotic pathway.

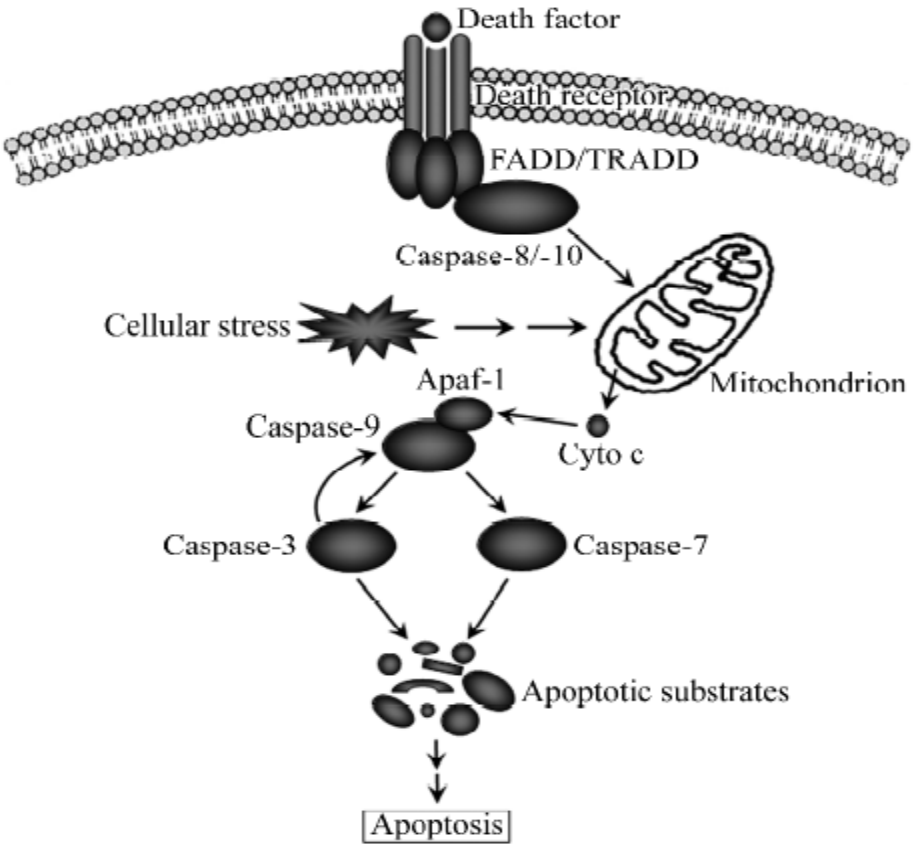


Figure 1.2 Apoptotic mitochondrial pathway (adapted from Fan *et al*., 2005)

The mitochondrial proapoptotic factors reside in mitochondrial intermembrane space (Kluck *et al.,* 1997), therefore a releasing mechanism is required. Two mechanisms have been suggested; the first one involves formation of a large channel in the outer mitochondrial membrane, due to oligemirisation of certain Bc1-2 homologous proteins (Gross *et al.*, 1998). Opening of the mitochondrial permeability transition pore (mPTP) is the second mechanism (Hunter & Haworth, 1979, Kromeor *et al.,* 1998). The mPTP refers to the massive swelling and depolarization of mitochondria due to opening of a non-specific pore in the inner mitochondrial membrane, known as mitochondrial permeability transition pore (mPTP) (Halestrap *et al,* 2002). The exact molecular composition of mPTP

is still under debate (Halestrap *et al.,* 2002). However, several studies describe it as a non-selective pore formed at the site of contact between the mitochondrial inner and outer membrane, and that the pore consists of a complex of the adenine nucleotide translocator (ANT), the mitochondrial voltage-dependent anion channel (VDAC), cyclophidine D, creatine kinase and possibly other proteins (Crompton 1999, Halestrap *et al.,* 2002, 2004). Several studies have shown that mPTP opens during reperfusion and not during ischemia (Crompton *et al.,* 1987, Griffith & Halestrap, 1995, Halestrap *et al.,* 2004). The probability of inducing the mPTP opening increased by the same factors that prevail in the setting of reperfusion injury. Pharmacological inhibition of mPTP is shown to be cardioprotective (*Nazareth et al.,* 1991, Griffiths & Halestrap 1993, Kroemer *et al.,* 1998). There is increasing evidence that almost any procedure that reduces reperfusion injury is associated with either a decrease in mPTP opening, or an increase in subsequent pore closure (Hausenloy *et al.,* 2002, Halestrap *et al.,* 2004).

Recently, a new pathway has been involved in apoptosis induction which is the endoplasmic reticulum (ER) stress-induced apoptotic pathway (Fan *et al.,* 2005). Following ER stress, leakage of Ca^{2+} from ER stores into the cytosol may have serious deleterious consequences. Irregular Ca^{2+} concentrations activate the Ca^{2+} dependent protease, calpain which is thought to process caspase-12 which then processes and activates other caspase to induce apoptosis. In addition, Ca^{2+} is the principle trigger of Mitochondrial Permeability Transition Pore (MPTP) opening (Duchen, 2000), which then initiates mitochondrial pathway (Figure 1.3). Therefore the mitochondria and ER are inextricably linked

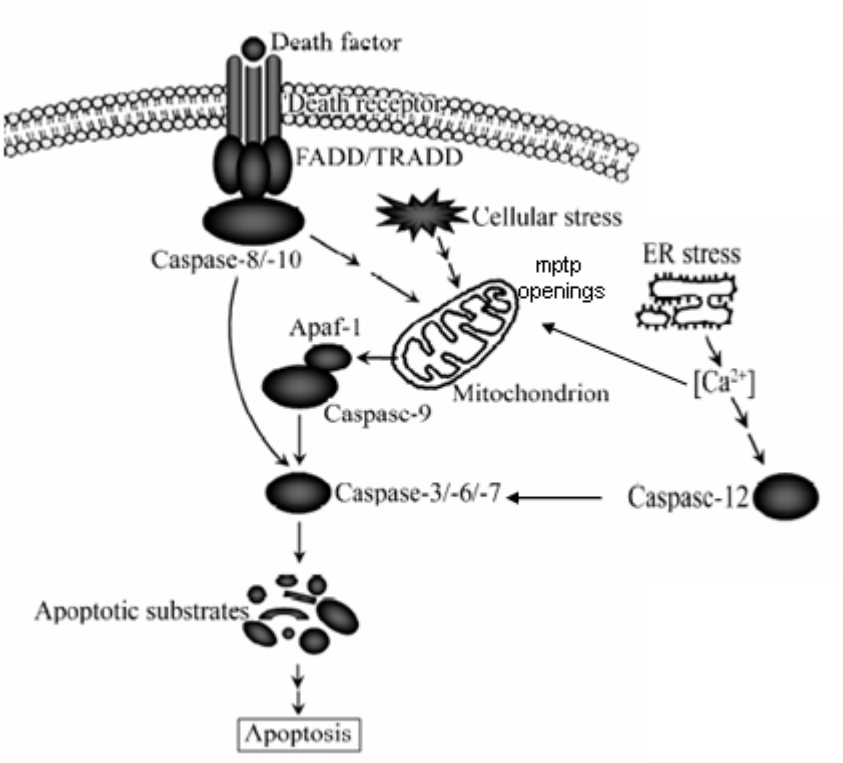


Figure 1.3 Endoplasmic reticulum (ER) stress-induced apoptotic pathway (adapted from Fan *et al.*, 2005)

From the above discussion, we can see a cross talk between the three pathways through their convergence at caspase 3 activation and through Bid as shown in Figure 1.3.

1.5 Caspase substrates during apoptosis

The enzyme poly (ADP-ribose) polymerase, or PARP, is an abundant nuclear protein. PARP is involved in repair of DNA damage and functions by catalyzing the synthesis of poly (ADP-ribose) and by binding to DNA strand breaks and modifying nuclear proteins. It was the first protein identified as a substrate for the executioner caspase 3 and 7 (Smulson *et al.*, 2000). The ability of PARP to repair DNA damage is prevented following cleavage by caspase and eventually DNA degradation will ensue (Smulson *et al.*, 2000). DNA

topoisomerase II is a nuclear enzyme essential for DNA replication and repair. Caspase can also inactivate this enzyme leading to DNA damage (Haung *et al.,* 2003).

The fragmentation of DNA into nucleosomal units - as seen in DNA laddering assays of apoptosis is caused by an enzyme known as CAD (caspase activated Deoxyribonuclease) (Enari *et al.,* 1998, Mukae *et al.,* 1998). Normally CAD exists as an inactive complex with ICAD (inhibitor of CAD, also known as DNA fragmentation factor45). During apoptosis, caspase-9 damages the nuclear pores in an unknown fashion, so that caspase-3 can enter the nucleus to cleave ICAD (Fan *et al.,* 2005). This will result in CAD release and rapid fragmentation of the nuclear DNA follows (Sakahira *et al.,* 1998).

Lamins are intra-nuclear proteins that maintain the shape of the nucleus and mediate interactions between chromatin and the nuclear membrane. Degradation of lamins by caspase 6 results in the chromatin condensation and nuclear fragmentation commonly observed in apoptotic cells (Ruchaud *et al.,* 2002). Fodrin is an essential nuclear and cytosolic skeleton and its cleavage by caspase-3 results in apoptotic body formation during apoptosis (Fan *et al.,* 2005). Communal *et al.,* (2002) also showed that caspase-3 cleaved myofibrillar proteins such as troponin T and actin, resulting in impaired force production and myofibrillar ATPase activity and eventually impaired myocardial contractile function.

Activation of caspase during apoptosis collectively results in a series of changes including further positive feedback activation of more caspase, DNA damage, decrease in DNA repair ability, cytoskeleton disassembly and chromatin condensation. The cell inevitably undergoes apoptosis. Therefore, caspase activation has been viewed as the “point of no

return" (Golstein, 1997) and inhibition of caspase could provide a valuable therapeutic approach in diseases where apoptosis play a significant role such as myocardial ischemia reperfusion injury.

1.6 Endogenous Caspase regulators and inhibitors

Endogenous caspase inhibitors have been identified that regulate caspase activity such as cFLIP (cellular FLICE-inhibitory protein), IAP (inhibitory apoptosis protein) and ARC (apoptosis repressor with caspase recruitment domain) (Gill *et al.,* 2002, Fan *et al.,* 2005).

As mentioned earlier Bc1-2 family proteins also played an important role in regulating the mitochondrial pathway of activating caspase. According to functional and structural criteria, the Bc1-2 members can be divided into two groups (Fan *et al.,* 2005). Group I proteins are all anti-apoptotic proteins such as Bcl-2 and Bcl-xL. The mechanism of their anti-apoptotic functions is via inhibition of proapoptotic proteins of the Bcl-2. Group II proteins are all proapoptotic proteins such as Bad, Bak Bax, Bcl-xS, Bid, Bik, and Bim. After apoptosis induction, Bax and Bak can translocate to the mitochondrial outer membrane where they undergo conformational changes, oligomerization and insertion into the mitochondrial outer membrane and eventually initiate the mitochondrial pathway as mentioned earlier. These proteins are involved in the setting of ischemia reperfusion injury where reactive oxygen species increase the expression of proapoptotic Bad and elicit translocation of Bax and Bad to the mitochondria, resulting in cytochrome *c* release, activation of caspase-3, and cleavage of PARP (von Harsdorf *et al.,* 1999). In addition overexpression of Bcl-2 in ventricular myocytes prevents apoptosis (Kirshenbaum *et al.,* 1997). Furthermore, cardioprotection by preconditioning (Transient non-lethal episodes of

myocardial ischemia and reperfusion confer protection against a subsequent lethal episode of prolonged myocardial ischemia) was found to be mediated through up-regulation of the antiapoptotic Bcl-2 pathway (Maulik *et al.,* 1999).

Calcium (Ca^{2+}) plays an important signaling role during apoptosis. It is well established that changes in intracellular Ca^{2+} are associated with reperfusion injury and apoptosis as discussed earlier. Numerous Ca^{2+}-regulated effectors have been identified such as Ca^{2+}/calmodulin-regulated phosphatase calcineurin, which promotes apoptosis through dephosphorylation of Bad, which then translocates to the mitochondria and effect cytochrome c release (Wang *et al.,* 1999). Ca^{2+}-dependent cysteine protease calpain is another regulatory protein. It is generally believed that calpain is activated in both necrosis and apoptosis (McGinnis *et al.,* 1999). Procaspase-12 activation is thought to be dependent on Ca^{2+} and calpains (Mandic *et al.,* 2003). Calpains also reported to cleave Bax, promoting its proapoptotic activity (Wood &Newcomb, 2000). In addition, calpains can also cleave Bid to an active fragment capable of mediating cytochrome c release and promoting apoptosis (Chen *et al.,* 2001). Calpain was found to first cleave procaspase-3 into 29 kDa fragments to facilitate its further cleavage and activation in the brain cells of rats suffering from unilateral hypoxia-ischemia (Blomgren *et al.,* 2001). Furthermore caspase-3 shares many common substrates with calpains including fodrin and PARP (Wang, 2000). Pharmacological inhibition of calpains has been found to reduce myocardial infarction size, apoptosis and improve function during ischemia repefusion injury (Yoshida *et al.,* 1995, Khalil *et al.,* 2005,).

1.7 Synthetic caspase inhibitors and myocardial ischemia reperfusion injury

Several caspase have been shown to be activated in the heart in response to ischemia reperfusion stimuli (Holly *et al.,* 1999) and their activation has been accelerated with reperfusion (Black *et al.,* 1998, Vanden Hoek *et al.,* 2002). The use of synthetic caspase inhibitors showed promising results in different studies of myocardial ischemia reperfusion. Broad spectrum caspase inhibitors significantly reduced myocardial infarct size (Yaoita *et al.,* 1998, Holly *et al.,* 1999, Mocanu *et al.,* 2000), which has been attributed to reduced apoptotic cell death. Specific caspase inhibitors also showed significant reduction in apoptotic cell death, however, their effect on reducing infarct size was different between studies. Okamura *et al.,* (2000) found that administration of caspase-1 and caspase-3 inhibitors 5 minutes before ischemia resulted in significant reduction in apoptotic cell death without significant change in infarct size. In contrast Mocanu *et al.,* (2000) found a significant reduction in infarct size after using broad spectrum caspase inhibitor (ZVAD) and caspase 8, 9 and 3 inhibitors, which were added at the time of reperfusion. This discrepancy in results could be attributed to differences in timing of administration of the caspase inhibitors.

The effect of delayed administration of caspase inhibitors after starting reperfusion on myocardial damage is still not known. This delayed administration is clinically more feasible as patients are usually presented to hospital after the onset of ischemic attack. Furthermore spontaneous reperfusion may occur in some patients before any hospital intervention due to dislodgment of the thrombus or relieved coronary spasm (Christian *et al.,* 1998). Therefore investigating the potential therapeutic window of caspase inhibitors

after the onset of reperfusion is considered to be important. Caspase inhibitors were found to be neuroprotective even when administered 9 hours after reperfusion using a brain ischemia/reperfusion model (Fink *et al.,* 1998), while others found that they were neuroprotective only when given only at the time of reperfusion (Hara *et al.,* 1997). Armstrong *et al.,* (2001) presented an abstract at the American Heart Association showing that a caspase inhibitor currently under investigation by scientists at Idun Pharmaceutical, Inc. (San Diego, CA), reduced infarct size up to 55% even when administered one hour after the heart attack using in *vivo* rat model. However Li *et al.,* (2001) using isolated adult rabbit cardiomyocytes showed that caspase inhibitors (unspecified, labeled with numbers) were partially protective if added 15 min after reperfusion and the effect was completely lost with a delay of 30 or 60 minutes. This discrepancy could be attributed to the different inhibitors used (both used unknown inhibitors identified by numbers) and different models used. Therefore further research is needed before applying caspase inhibitors in the clinical field.

The effect of caspase inhibitors on post ischemic functional recovery showed mixed results. Some studies showed functional recovery improvement after treatment with caspase inhibitors (Kovac *et al.,* 2001, Balsam *et al.,* 2005), where others showed non significant change in functional parameters (Perrin *et al.,* 2003, Holly *et al.,* 1999, Mocanu *et al.,* 2000). This controversy between studies could be attributed to the use of different inhibitors, different functional parameters, different ischemia reperfusion models and different timing of caspase inhibitors administration.

Specificity of synthetic caspase inhibitors should be taken with extreme care even though they have been reported to be specific by manufacturing companies. Sufficiency of a P4-P1 tetrapeptide for caspase recognition and high-affinity binding has been the platform for most of the currently available caspase inhibitors (Nicholson, 1999). Virtually, all of the current commercially available caspase inhibitors and probes have been designed based on the reported optimal substrate specificity profiles for the caspase (Thornberry *et al*., 1997). However, these primary peptide sequences may provide only limited selectivity when used in the context of a small molecule inhibitor. Berger et al., (2006) showed that none of the inhibitors compounds they tested are highly selective and all exhibit broad inhibition when used at high concentrations. Furthermore, they showed that it is possible that other "selective" caspase inhibitors designed to target other caspase may demonstrate similar cross-reactivity when used at concentrations required for cell-based studies. The similar significant cardioprotection offered by the broad spectrum and selective caspase inhibitors found by Mocanu *et al.*, (2000) raises the possibility that these compounds might have non-specific actions. The caspase inhibitors might have an inhibitory effect on other proteases implicated in myocardial ischemia reperfusion injury such as calpains. Calpains, which are also cysteine proteases and share many common substrates with caspase 3 (Wang, 2000), are involved in both necrotic and apoptotic cell death during myocardial ischemia reperfusion injury (McGinnis et *al*., 1999). Calpain inhibitors have also been shown to reduce myocardial ischemia reperfusion injury (Perrin *et al*., 2003, Khalil *et al*., 2005). In addition caspase inhibitors have also been found to efficiently inhibit cathepsin B activity *in vitro* and in tissue culture cells (Schotte *et al*., 1998, Gray *et al*., 2001), which is another cystein protease enzyme that has already been implicated in some apoptotic models

(Guicciardi *et al*., 2000, Stoka *et al*., 2001) and myocardial ischemia reperfusion injury (Tsuchida *et al*., 1986). Therefore non-caspase related mechanisms should be considered when interpreting the effect of caspase inhibitors.

In conclusion synthetic caspase inhibitors provide a promising route for development of therapies to attenuate reperfusion-induced injury in the heart. However, additional research is needed to further investigate the role of caspase inhibitors in reducing myocardial damage during reperfusion post ischemic injury before applying them in the clinical field. The optimum timing of treatment with caspase inhibitors and whether delayed administration of caspase inhibitors will be cardioprotective, is still not known. The long term effect of caspase inhibitors is not known yet. Whether caspase inhibition will result in long term tissue salvage or only short lived cardioprotection. Their effect on functional recovery of the myocardium following ischemia reperfusion is still controversial. The underlying mechanism of their action and their specificity is still not cleared by pervious investigations.

1.8 Ischemic preconditioning and postconditioning

Ischemic preconditioning firstly described by Murry *et al.,* (1986), whereby episodes of intermittent sublethal ischemia (conditioning ischemia) and reperfusion confer resistance against a subsequent lethal episode of myocardial ischemia using dog model. The same phenomenon has been described using different animal models (Schott *et al.,* 1990, Liu *et al.,* 1991) and also using human models (Walker *et al.,* 1995, Kloner *et al.,* 2002). The preconditioning phenomenon reported to have different cardioprotective effects including:

reduction in infarct size (Murry *et al.,* 1986), preservation of vascular endothelial function (Thourani *et al.*, 1999), decrease in polymorphonuclear neutrophils (PMNs) accumulation (Nakamura *et al*, 2000), and reduction in the appearance of apoptosis (Gottlieb *et al.,* 1996, Nakamura *et al.,* 2000).

An overwhelming number of studies have investigated the underlying mechanisms and the signalling cascades involved. Knowledge of these signalling cascades is essential to understand how various drugs could mimic ischemic preconditioning or interfere with it. During the pre-conditioning ischaemia, the heart releases several trigger substances including adenosine (Liu *et al.,* 1991), bradykinin (Goto *et al.,* 1992) and opioids (Schultz *et al* 1998). These three ligands occupy their respective receptors (GPCRs) which ultimately work in parallel to activate PKC. PKC has long been known to play a central role in preconditioning (Ytrehus *et al*., 1994).The pathway by which each of these ligands activates PKC is quite different, as shown in Figure 1.4. Bradykinin and opioid both activate osphatidylinositol 3-kinase (PI3K), which causes phosphorylation and activation of Akt. Akt phosphorylates endothelial NO synthase (eNOS), causing it to generate NO. NO activates soluble guanylyl cyclase (GC) causing it to make cGMP which then activates protein kinase G (PKG). PKG acts on mitochondria, causing opening of ATP-mitochondrial sensitive potassium channel on the inner membrane. Opening of mitochondrial ATP-sensitive potassium channel has two known actions: swelling of the matrix and generation of reactive oxygen species (ROS). ROS can directly activate PKC. Protection from a pulse of bradykinin or opioid can be blocked by co-administration of a PI3K inhibitor, eNOS inhibitor, GC inhibitor, mitochondrial ATP-sensitive potassium

channel blocker, or a ROS scavenger (Costa *et al*., 2008). Adenosine mediated cardioprotection found also to be mediated through activation of PI3K pathway where it appears to have a second coupling to PKC (Costa *et al*., 2005) as shown in figure 1.4. After this triggering phase, an intracellular signalling cascade through activation of PI3K, MEK1/2 – erk1/2 and PKC (Hausenloy *et al*., 2005) finally brings the cell into its protected phenotype through convergence on mitochondrial end effectors including ATP-sensitive potassium channel (Gross *et al.*, 1992) and the mitochondrial permeability transition pore (Hausenloy *et al.,* 2004) which protects the cardiomyocytes against both necrotic and apoptotic cell death (Figure 1.4).

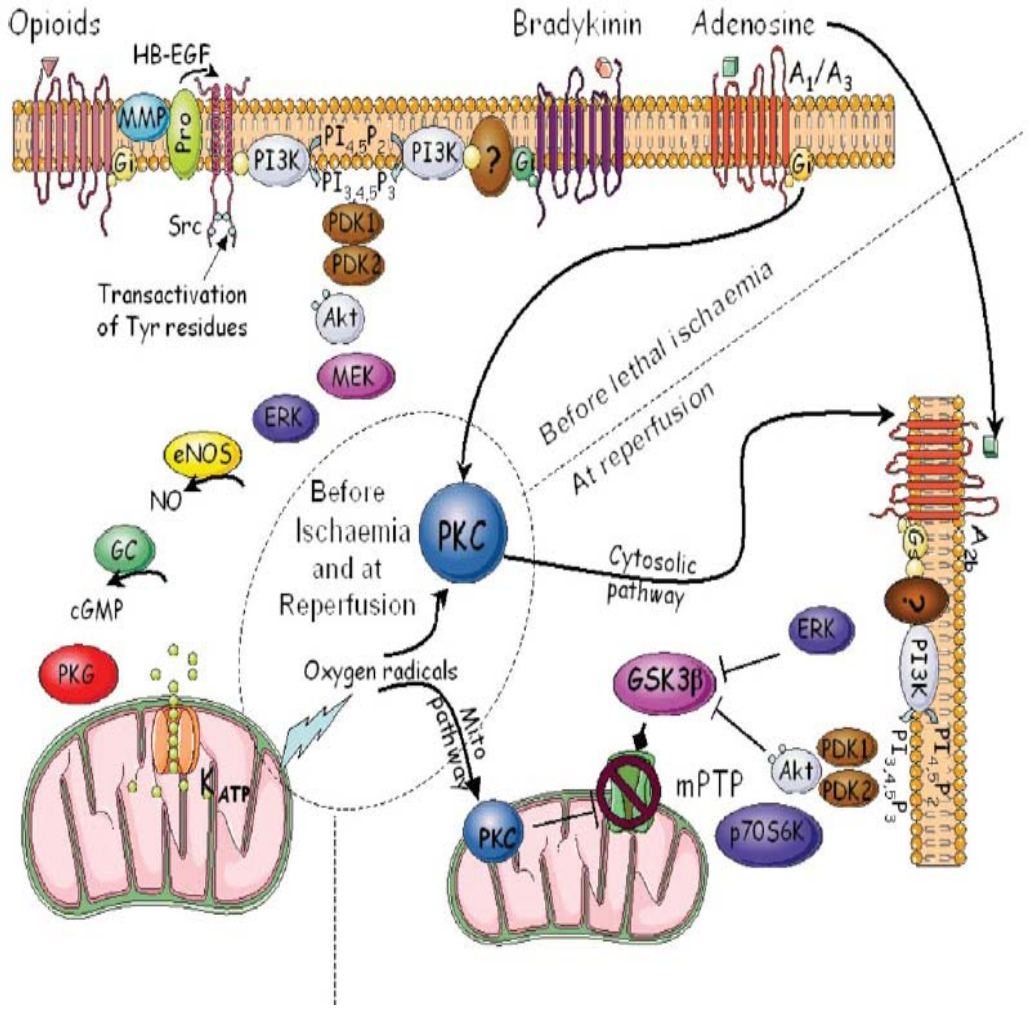


Figure 1.4 Simplified siganling pathways of myocardial preconditioning. MMP; matrix metalloproteinases; HB-EGF, heparin-binding epidermal growth factor-like growth factor; Pro, pro-HB-EGF; PDK, phospholipid-dependent kinase; PI3K, phosphatidylinositol 3-kinase; PI4,5P2, phosphatidylinositol bisphosphate; PI3,4,5P3, phosphatidylinositol trisphosphate; MEK, mitogen activated protein kinase; ERK, extracellular-signal regulated kinase; NO, nitric oxide; NOS, NOS synthase; eNOS, endothelial NOS; GC, guanylyl cyclase; PKG, protein kinase G; PKC, protein kinase C; KATP, mitochondrial ATP-dependent potassium channel; p70S6K, p70S6 kinase; GSK-3b, glycogen synthase kinase-3b; MPT, mitochondrial permeability transition (adapted from Costa *et al*., 2008).

Although, preconditioning has been clinically successful in attenuating the ischemia reperfusion injury in setting of percutaneous transluminal coronary angioplasty (Deutsch *et*

al., 1990, Tomai *et al.*, 2001), its practical use in clinical arena is limited by the inability to predict the onset of ischemia, which is the case in patients presented with myocardial infarction. However, the implementation of cardioprotective therapy at the time of reperfusion is clinically more feasible because the onset of reperfusion is more predictable and is under the clinician's control. In this respect, the newly described phenomenon of ischemic postconditioning, in which the application of intermittent episodes of myocardial ischemia and reperfusion at end of the index ischemic period confer a cardioprotective effect similar to ischemic preconditioning (Zhao *et al.*, 2003), provides one such intervention.

Postconditioning has been shown to reduce infarct size (Zhao *et al.*, 2003), reduce endothelial activation, dysfunction and neutrophil adherence (Zhao *et al.*, 2003) and significantly reduce apoptotic cell death (Zhao *et al.*, 2005). These cardioprotective effects found to be mediated through recruitment of signal transduction pathways as is the case in ischemic preconditioning. Recent studies suggest that ischemic postconditioning protects the myocardium by activating the PI3K–Akt pathway (Tsang *et al.*, 2004, Bopassa *et al.*, 2005, Yang *et al.*, 2005) and MEK1/2-erk1/2 pathway (Darling *et al.*, 2005, Yang *et al.*, 2004) at the time of myocardial reperfusion. Adenosine has been found to be one of the triggering mediators for activation of these signalling cascades (Kin *et al.*, 2004). Mitochondrial permeability transition pore believed to be the potential end effector mediating postconditioning cardioprotection (Argaud *et al.*, 2005) Figure 1.5.

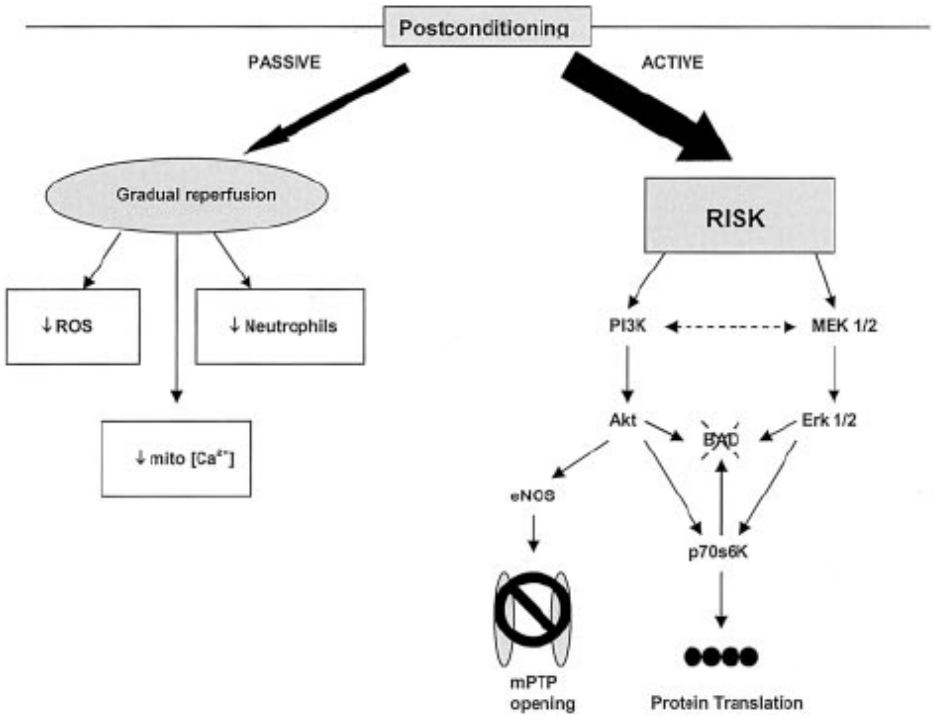


Figure 1.5. Schematic representation of the key mediators involved in ischaemic postconditioning mediated cardioprotection. (Adapted from Tsang et al., 2004).

Although, two clinical studies demonstrated that ischemic postconditioning is an efficient cardioprotective intervention for treatment of reperfusion injury in patients with ischemic heart disease (Laskey, 2005, Staat *et al.*, 2005), reintroduction of ischemia at the time of reperfusion may lead to potential complications (coronary plaque rupture and resultant restenosis and emboli) and also it is not clinically feasible during thrombolytic drug therapy. However, the concept of "pharmacological postconditioning", where pharmacological agents that mimic the effect of ischemic postconditioing by upregulation of survival signaling pathways is a more feasible way. Insulin (Jonassen *et al.*, 2001), Insulin like growth factor (Parrizas *et al.*, 1997), Urocortin (Brar *et al* 2000, Schulman *et al* 2002), Fibroblast growth factor (Cuevas *et al.*, 1997), Braykinin (Li & Sato, 2001) and

adenosine agonists (Schulte & Fredholm, 2000) has been used adjunctively to reperfusion therapy. Their cardioprotective effects found to be mediated through upregulation of survival signalling pathways, which mimics postconditioning cardioprotective mechanism. Therefore it is interesting to investigate whether cardioprotective agents introduced at the time of reperfusion exerts their effects through upregulation of the survival signalling pathways in a similar mechanism as ischemic postconditioning.

1.9 Pro-survival Kinase cascades

Several protein kinases have been implicated in the setting of ischemia reperfusion injury, which have been involved in both, the upstream induction phase of apoptosis and downstream execution phase of apoptosis (Cross *et al.*, 2000). Some kinase pathways play a pro-apoptotic role like c-Jun NH_2-terminal protein kinase (JNK), while others play a pro-survival role such as phosphatidylinositol-3-OH kinase (PI3K)–Akt and p42/p44 extra-cellular signal-regulated kinases (Erk 1/2) (Hausenloy & Yellon, 2004). However the role of p38 mitogen-activated protein kinase (MAPK) in apoptosis is a matter of debate. In endothelial cells, some researchers have shown that induction of apoptosis by treatment with thrombospondin-1 (Jimenez *et al*., 2000) or high levels of D-glucose (Nakagami *et al*., 2001) requires p38 MAPK activity, whereas others have reported that inhibition of apoptosis by estradiol is mediated via activation of p38 MAPK (Razandi *et al*., 2000). Therefore there is accumulating evidence that the involvement of p38 MAPK in apoptosis is both cell-type and stimulus dependent (Grethe *et al*., 2004).

The PI3K–Akt signalling cascade is activated in response to the activation of a wide range of receptors, including those for growth factors and G-protein-coupled receptors (Cross *et al.,* 2000). Signalling through PI3 kinase has been demonstrated to confer protection against ischemia–reperfusion injury (Matsui *et al.,* 1999), through its activation of the serine–threonine kinase, Akt (Datta *et al.,* 1996). The Erk 1/2 or p42/p44 signalling cascade is a member of the mitogen-activated protein kinases (MAPKs), which have been shown to be activated in the setting of ischemia–reperfusion, and also can mediate cellular protection (Shimizu *et al.,* 1998, Yue *et al.,* 2000). These two pathways have been shown to mediate cardioprotection induced by different growth factors and pharmacological agents introduced at the time of reperfusion such as Insulin (Jonassen *et al.,* 2001), Insulin like growth factor (Parrizas *et al.,* 1997), Urocortin (Brar *et al.,* 2000, Schulman *et al.,* 2002), Fibroblast growth factor (Cuevas *et al.,* 1997), Bradykinin (Li & Sato, 2001) and adenosine agonists (Schulte & Fredholm, 2000). In addition, the cardioprotection offered by ischemic preconditioning is mediated by up-regulation of PI3-Akt and Erk1/2 signalling cascades at the time of reperfusion (Hausenloy *et al.,* 2003). Furthermore, the cardioprotection induced by post conditioning also found to be mediated by upregulation of these two pathways (Hausenloy & Yellon, 2006). Recently, Hausenloy *et al.,* (2004) have proposed a cross talk between the PI3K and the Erk1/2 during early reperfusion. They showed that blockade of the PI3K activity upregulated ERK1/2 phosphorylation and blockade of ERK1/2 upregulated Akt phosphorylation.

Most studies suggested that the cardioprotection provided by PI3-Akt and Erk1/2 signalling pathways could be attributed to their ability to phosphorylate and inactivate the pro-

apoptotic proteins. Phosphorylation of BAD (pro-apoptotic protein) by activated PI3-Akt or the MEK1/2-Erk1/2 results in its binding to 14-3-3 proteins, which prevent it from binding to its mitochondrial target, therefore preventing apoptosis (Zha *et al.*, 1997). In addition activation of these pro-survival pathways inhibits the conformational change of Bax (pro-apoptotic protein) required for its translocation to mitochondria, thereby preventing apoptosis (Yamaguchi & Wang, 2001). Another study also showed that inhibition of either PI3-Akt or Erk1/2 resulted in an increase in BIM (pro-apoptotic protein) expression (Weston *et al.,* 2003). Furthermore activation of the PI3-Akt pathway was found to target other proapototic proteins like P53 through phosphorylation of Mdm2 (Mayo & Donner, 2001).

At the mitochondrial level of apoptosis, PI3-Akt activation was able to inhibit mitochondrial cytochrome c release and maintain mitochondrial membrane potential, independent of BAD phosphorylation (Kennedy *et al.,* 1999). This could be through phosphorylation of eNOS (Balakirev *et al.,* 1997). AKT has been shown to phosphorylate eNOS, which will produce nitric oxide, which has been shown to inhibit opening of the mPTP (Balakirev *et al.,* 1997). However, recently Davidson *et al.,* (2006) showed that activation of the PI3K-Akt pro-survival kinase pathway inhibits opening of the mPTP, and demonstrated an important link between the survival kinases and the mPTP. In addition, signalling through PI3-Akt pathway has been shown to activate protein kinase C, which mediates the cellular protection associated with preconditioning (Yellon & Downey, 2003).

Downstream at the caspase level of apoptosis, Akt activation has been found to phosphorylate and inactivate pro-caspase 9, and thereby inhibiting apoptosis (Cardone *et al.,* 1998). Ehardt *et al.,* (1999) found that addition of cytochrome c to cytosol of cells over-expressing B-Raf (which results in activation of Erk1/2) failed to induce caspase activation, which suggest that the B-Raf-MEK/Erk pathway confers protection against apoptosis at the level of cytosolic caspase activation, downstream of release of cytochrome c from mitochondria. Furthermore Erk1/2 kinase activation in haematopoietic cells was found to inhibit apoptosis through inhibition of caspase-3 activation (Terada *et al.,* 2000).

Akt and Erk pathways cardioprotective effects have been shown to be mediated through gene expression regulation. Akt phosphorylates and activates IKK-α, which leads to activation and translocation of NF-κB, where it acts as a transcription factor for a variety of survival pathways (Romashkova &. Makarov, 1999) Akt activation was also found to phosphorylate Forkhead transcription factor FKHRL1 by sequestering it in the cytosol and preventing FKHRL1-mediated transcription of death inducing genes such as Fas ligands (Brunet *et al.,* 1999). Erk activation has been found to phosphorylate p90RSK, which is linked to regulation of proteins concerned with genes concerned with cellular survival (Bonni *et al.,* 1999).

Therefore, it is apparent that therapeutic interventions which target and activate the PI3-Akt and Erk1/2 cell signalling pathways during the early reperfusion phase can be used as an adjunct to current reperfusion therapy, and may provide a new therapeutic approach to limit

infarction size and maintain myocardial viability in patients presenting with an acute myocardial infarction.

Synthetic caspase inhibitors have been shown to be cardioprotective when administered during early phase of reperfusion (Haung *et al.*, 2000, Mocanu *et al.*, 2000); however the precise mechanism behind their cardioprotection is not yet clear. Caspase can be activated by two main pathways, the cell surface death receptors and mitochondrial pathway (for details check section 1.4). The caspase inhibitors failed to inhibit cytochrome c release from mitochondria as showed by previous studies, which suggests that cytochrome c release is upstream and independent of caspase activation (Bialik *et al.*, 1999, de Moissac *at al*., 1999, Suzuki *et al*., 2001). Therefore, caspase inhibitors mediated cardioprotection is not dependent on mitochondrial cytochrome c pathway and an alternative mechanism operating at the level of cytosolic caspase activation can be suggested. This theory can be supported by the fact that both the broad and the specific caspase inhibitors were effective to approximately the same degree (Mocanu *et al.*, 2000), which raised the possibility that they might share simillar pathway. As mentioned above cadioprotection by pharmacological agents that mimic preconditioning effect and drugs applied during the early phase of reperfusion were mediated through activation of PI3-Akt and Erk 1/2 cell signalling pathways. Therefore, it appeared that activation of these survival kinase pathways may constitute a common survival pathway sufficient to induce a cardio-protective response during early reperfusion phase (Hausenloy & Yellon, 2004). Caspase inhibitors showed a promising cardioprotection when applied during early phase of

reperfusion, which could also be hypothesized to be mediated by activation of these cell survival pathways.

Wu *et al*., (2000) showed a novel link between PI3-kinase pathway and suppression of caspase 3 activation independent of Bad phosphorylation using cardiac muscle cells. Akt pathway also has been found to inhibit caspase mediated apoptosis through direct phosphorylation of caspase 9 (Cardone *et al*., 1998). Furthermore Widmann *et al* (1998) demonstrated that active caspase turn off survival signals by degredation of Akt and other signalling proteins using Jurkat and U937 cell model. Therfore caspase inhibitors cardioprotection during reperfusion could be hypothesised to be mediated through PI3-Akt pathway

1.10 Experimental models for the study of myocardial ischemia reperfusion injury

In the arena of cardiovascular research, the ultimate aim of the researchers is to improve the understanding of the mechanisms underlying normal cardiovascular function and enhancing the ability to understand and ameliorate cardiovascular disease. Ischemic heart diseases is an increasing worldwide health problem, therefore developing an experimental models to study the underlying pathophysiology of ischemia and reperfusion injury, important pathways involved in mediating cell death and the efficacy of pharmacological interventions considered important.

In assessing the utility of an investigative model, it is useful to identify the endpoints which can be used either as a measure of the normal function of the organ under study or of the disease which is afflicting it. In the case of the heart research, the main endpoints are: 1) cardiac size, shape and growth, 2) its pump function via a host of hemodynamic, contractile or work indices, 3) heart rate, rhythm and electrical activity, 4) biochemistry via substrate uptake or the many aspects of cellular metabolism and its regulation, 5) pharmacological responsiveness, particularly to drugs influencing contractile, vascular or electrical activity and cellular death, 6) ultrastructure, 7) excitation-contraction coupling and ion regulation, and 8) the regulation and expression of the genetic machinery of the cell (Hearse & Sutherland, 2000). Therefore, the choice of a good experimental model depends on the ability to balance between the quality of measured endpoints and their clinical relevance. In addition, the choice of the model depends heavily on the problem under examination, and the hypothesis that is to be tested.

In general human tissue models are the more clinically relevant, however they are not easily available and costly. Results obtained from animal experimental models of the ischemic heart have been of tremendous importance in understanding ischemia reperfusion injury and contributes significantly to future development of therapeutic interventions. Experimental animal models in use for the study of the ischemic heart involve studies on the integrated organism, experiments with isolated hearts or multicellular preparation, and also studies of cells isolated from the heart. This review will focus on the different animal experimental models used in this study and their advantages and disadvantages.

1.10.1 Isolated perfused heart-Langendorff perfusion system

Langendorff (1895) was the first one to describe the isolated perfused heart preparation. It probably represents the optimal compromise in the conflict between the quantity and quality of data that can be acquired from an experimental model and its clinical relevance, especially in relation to the modelling of ischemia (Sutherland & Hearse, 2000). The basic principle of this model is rapid removal of the heart from the animal and thereafter mounting in a langendorff perfusion system with a perfusion solution (i.e. Krebs–Henseleits bicarbonate buffer gassed with 5% CO2 and 95% O2) that secures a supply of energy substrate. It involves the cannulation of the aorta to the langendorff perfusion system containing oxygenated perfusion fluid. This fluid is then delivered in a retrograde direction down the aorta either at a constant flow rate (delivered by an infusion or roller pump) or a constant hydrostatic pressure (usually in the range of 60-100 mmHg). In both instances, the aortic valves are forced shut and the perfusion fluid is directed into the coronary ostia thereby perfusing the entire ventricular mass of the heart, draining into the right atrium via the coronary sinus (Sutherland & Hearse, 2000).The left ventricular heart chamber is empty or sparsely filled with fluid, therefore the preparation performs minimal external work. Different modes of ischemic conditions can be induced, global ischemia is caused by clamping the aortic inflow, hypoxia by reducing oxygen supply to the perfusion solution and regional ischemia by a ligature around a coronary artery (Ytreuhs, 2000).

At a practical level, the isolated heart, especially from small mammals, provides a highly reproducible preparation which can be studied quickly and in large numbers at relatively

low cost. Furthermore, the isolated heart preparation allows experiments to be continued in the face of events such as infarction-induced loss of pump function, cardiac arrest or arrhythmias, which would normally jeopardise the survival of an *in vivo* experiment (Sutherland & Hearse, 2000). It has been a first-choice model for studies of the metabolic, biochemical and cellular consequences of myocardial ischemia through venous effluent and myocardial tissue samples (conserved by freeze-clamping in liquid nitrogen) which can easily be obtained (Ytreuhs, 2000). These studies can be made in the absence of the confounding effects of other organs, the systemic circulation and a host of peripheral complications such as circulating neurohormonal factors. This model with the aid of conventional histochemistry techniques provides a valuable method to study the ischemic effect on infarct size, which represents the ultimate endpoint of the ischemic process (Ytreuhs, 2000).

An intraventricular balloon connected to a pressure transducer is used for measurements of contractile function. Left ventricular systolic, diastolic, developed pressure and heart rate can be recorded. This provides provision of baseline data and helps to monitor the stability of the heart and the extent of any disturbances of cardiac rhythm. However the isolated heart working langendorff model allows measurement of cardiac output with different filling pressures and afterloads which provides more sensitive parameters of contractile function (Ytreuhs, 2000).

One of the main disadvantages of isolated heart-langendorff model is that it is a constantly deteriorating preparation and able to maintain cellular haemostasis only for limited time.

The use of intraventricular balloon might cause ischemia induced necrosis to papillary muscle as it is not always well perfused. The perfusion solution is lacking blood cells, plasma proteins, growth factors and hormones which lead to changes in vascular function independent of the ischemia-induced changes in vascular function. Conventional histology for ischemia-induced necrosis is not possible with this model (Ytreuhs, 2000).

1.10.2 Multicellular tissue model

Multi-cellular preparations are mostly papillary muscle or atria trabecula muscle harvested from different species and superfused with buffer. The main endpoints that can be measured using this model are contractile force, electrical conduction and enzyme release (Ytreuhs, 2000).

The main advantages of this model is when the supply of heart tissue is very limited (an important example is the use of human atria trabecula) and also allows expensive molecular probes to be used since both tissue size and volume of superfusate are limited (Ytreuhs, 2000). The disadvantage of this model is spontaneous deterioration of the tissue due to inappropriate oxygenation. In some experimental conditions there is a need to use temperatures below 37 ^{o}C and electrical stimulation rates below the heart rate of the actual species under *in vivo* conditions which makes it further away from *in vivo* state (Ytreuhs, 2000).

1.10.3 Cardiomyocyte Model

Cardiomyocytes have been successfully isolated from hearts of all species and maintained in suspension for hours. Both suspensions and cultures of cardiomyocytes have been used in ischemia reperfusion studies. With cellular model it is almost impossible to create conditions similar to ischemia, however ischemia can be simulated by manipulation of the incubation buffer, hypoxia, or pelleting and sealing under paraffin. The time course of ischemic injury in cell models is usually substantially extended compared to intact tissue (Ytreuhs, 2000).

In this model there is a lack of attachment conditions and communication between isolated cardiomyocytes and other types of cells and compounds in the interstitium. This creates immense difference in living conditions between an isolated myocyte and the same cell as a part of an organism. Another disadvantage of this model is that spontaneous deterioration of myocytes will always be superimposed on the deterioration caused by simulated ischemia (Ytreuhs, 2000).

Using this cellular model, several possible endpoints for quantification of hypoxic cell injury and death exists. This model also allows clarifying whether a response to ischemia can be pinpointed to a single cell type. In this study, this model used to quantify the differential contribution of different types of cell death (apoptosis and necrosis) in response to ischemia reperfusion injury. Also this model allowed us to measure the intracellular

caspase-3 activity level which further expands our results about cardiomyocytes response to ischemia reperfusion injury and pharmacological treatment.

1.11 Methods to detect apoptosis

In order to evaluate the importance and contribution of apoptosis in different pathological diseases, it is critical to be able to quantitatively and qualitatively measure apoptosis. There are various approaches currently being used to measure apoptotic cell death based on cellular and biochemical changes during apoptosis (Loo & Rillema, 1998).

1.11.1 Cell morphology

The standard morphological features of apoptosis are best seen by electron microscopy, but can be also observed at the light microscopic level using nucleic acid-binding dyes, such as haematoxylin, acridine orange, or propidium iodide (Coles *et al*., 1993, Hall *et al*., 1994). A cell undergoing apoptosis proceeds through various stages of morphological changes. These are shrinkage of the cell away from its neighbors, plasma membrane blebbing, cytoplasmic and nuclear condensation, non-random cleavage of chromatin, margination of chromatin in the nucleus, nuclear fragmentation, and cellular fragmentation into smaller apoptotic bodies (Kerr *et al*., 1972). Apoptotic bodies are generally phagocytosed by surrounding cells. Therefore, the most common sign of apoptosis in a tissue section is the presence of apoptotic bodies, which may be seen as extracellular bodies, or, after phagocytosis, inside other cells (Wyllie *et al*., 1984). Apoptotic bodies have a diverse appearance, particularly in regard to their size. They are generally oval or round in shape,

and are most easily recognised when they contain large amounts of homogeneous, condensed chromatin (Kerr *et al*., 1972).

This method provides the most definitive morphological evidence of apoptosis; however, it does not provide quantitative data, and thus is not an appropriate method to quantify apoptosis between experimental conditions or over the time course of an experimental treatment (Loo & Rillema, 1998).

1.11.2 DNA fragmentation

During apoptosis the genomic DNA is cleaved into internucleosomal DNA segments by an endonuclease selectively activated during apoptosis (Wyllie, 1980). Internucleosomal DNA fragmentation appears to be preceded by the formation of larger DNA fragments with lengths of 50 and 300 kb (Wyllie, 1980). Separation of cellular DNA on agarose gels shows a characteristic ladderlike pattern of fragments with multiples of 200 bp in length (DNA laddering) (Loo & Rillema, 1998). However, this technique is not specific, since such analysis requires the extraction of DNA from a large numbers of cells which can be contaminated with other non-cardiomyocytes, whose number exceeds cardiomyocyte in myocardial tissue (Loo & Rillema, 1998). Therefore, other techniques have been developed based on detection of DNA strand breaks (‘3-OH ends). This is done through addition of certain enzymes that can add labeled nucleotides to the DNA ends. The labeled nucleotides can then be identified by immunological methods akin to immunohistochemistry. One of these methods termed TUNEL (terminal deoxynucleotidyl transferase mediated UTP nick end labeling). This method utilizes the activity of terminal deoxynucleotidyl transferase

(TdT) enzyme to incorporate biotinylated dUTP onto 3' ends of fragmented DNA (Gavrieli *et al*., 1992). The other method called in situ end labeling (ISEL), which relies on the activity of DNA polymerase I to fill in recessed 3' ends of DNA fragments with biotinylated-dUTP (Ansari *et al*., 1993). These methods are highly sensitive for apoptosis detection, however they are not specific. This because '3-OH DNA ends is not a unique feature of apoptosis and may occur during DNA repair and non-specific DNA damage (Eastman & Barry, 1992). Other disadvantage of these methods is that, not all cells undergoing apoptosis exhibit measurable internucleosomal DNA fragmentation (Loo & Rillema, 1998).

1.11.3 Cell membrane alterations

In normal cells the distribution of phospholipids is asymmetric with the inner membrane containing anionic phospholipids, such as phosphotidyl serine (PS) and the outer membrane having mostly neutral phospholipids. In apoptotic cells, the amount of PS on the outer surface of the membrane increases exposing PS to the surrounding liquid (Martin *et al*., 1995). Annexin V, a calcium dependent phospholipids binding protein, has a high affinity for PS. Annexin V binds to PS in the outer membrane of apoptotic cells, thus providing a suitable way of detecting apoptosis (Martin *et al*., 1995). Translocation of PS to the external cell surface is not unique to apoptosis, but occurs also during cell necrosis. The difference between these two forms of cell death is that during the initial stages of apoptosis the cell membrane remains intact, while at the very moment that necrosis occurs the cell membrane looses its integrity and becomes leaky. Therefore the measurement of Annexin V binding to the cell surface as indicative for apoptosis has to be performed in

conjunction with a dye exclusion test to establish integrity of the cell membrane (Vermes *et al*., 1995).

Flow cytometry can be utilized for quantitative analysis of the number of viable, apoptotic and necrotic cells by the rate of uptake and retention of certain dyes. Vermes *et al*., (1995) developed a novel assay for apoptosis flow cytometric detection of PS expression on early apoptotic cells using fluorescein labelled Annexin V. Simultaneously with dye exclusion technique (e.g propidium iodide, PI) and a dye to stain viable cells, this test will enable quantitative discriminative measurement of cellular apoptosis, necrosis and viable cells. This assay is rapid, however its sensitivity is time dependent and don't detect early apoptotic cells (Vermes *et al*., 2000).

1.11.4 Apoptosis induced caspase

Caspase activation is a cellular event associated with the onset of apoptotic death. Detection of caspase activity provides a useful assay for analyzing one of the earliest known biochemical events associated with apoptosis. Determination of caspase activation can be performed in different ways. This includes the analysis of pro-caspase processing using the immunoblot technique, analysis of enzyme activity by the cleavage of synthetic substrates, affinity labeling of activated caspase and immunoblot analysis of the cleavage of caspase substrates (Kohler *et al*., 2002). Recently a flow cytometry asssays has been developed to measure intracellular caspase activity (Komoriya *et al*, 2000). Caspase-3 is a key effector caspase in the apoptosis process. The active caspase-3 proteolytically cleaves and activates

other caspase as well as relevant targets in the cell like poly (ADP-ribose) polymerase (PARP) in the nucleus as mentioned earlier. Nicholson *et al*., (1995) have identified the tetrapeptide Asp–Glu–Val–Asp (DEVD) as a consensus cleavage site for caspase-3 through mapping of the cleavage site of PARP. Based on this information Polyclonal antibodies recognizing only the active form of caspase-3 has been developed and formed the basis of flow cytometry techniques to demonstrate intracellular caspase-3 activity. These antibodies were made against an active human caspase-3 fragment and bind to a conformational epitope which is exposed by activation-induced cleavage of pro-caspase-3. By using specific caspase-3 fluorogenic substrate, one can demonstrate intracellular caspase-3 by flowcytometry (Vermes *et al*., 2000).

The estimation of activated caspase in whole cells by flow cytometry has the advantage that it is possible to identify, and even separate, the quantity of the cells in a population, which contain activated caspase. This cannot be achieved by analysis of cell lysates used to calculate the total amount of activated caspase in a cell population. Furthermore, it is a quantitative method having the advantage of speed and sensitivity with small samples. The availability of a reliable antibody is the main limiting factor in this method (Kohler *et al*., 2002).

As seen above, there are a lot of methods that can be used to detect apoptosis; however, none of them can be considered as highly specific and sensitive. Therefore combination of more than one method is advised to achieve high accuracy in measuring apoptosis. The choice will depend on the experimental model, on cell type and researcher experience. In

addition it is important to apply a rigorous quantitation process through out the intended project to reach accurate results.

CHAPTER 2

2.1 Aims

1. To determine whether the broad spectrum caspase inhibitor (ZVAD) and specific caspase 3 inhibitor (DEVD) when administered at different time points during reperfusion (at the start of reperfusion, 15 minutes, 30 minutes and 60 minutes after starting reperfusion) protects the myocardium from ischemia reperfusion injury using the Langendorff ischemic-reperfused heart model.

2. To determine whether the broad spectrum caspase inhibitor (ZVAD) and specific caspase 3 inhibitor (DEVD) can attenuate apoptosis, necrosis and caspase-3 activity when administered at different time points during reoxygenation (at the start of reoxygenation, 15 minutes, 30 minutes and 60 minutes after starting reoxygenation) using the Isolated adult rat ventricular myocytes model of ischemia and reperfusion.

3. To determine whether PI3-Akt cell survival pathway is involved in the aforementioned caspase inhibitors mediated cardioprotection using a specific Akt inhibitor (Wortmanin).

4. To determine whether the broad spectrum caspase inhibitor (ZVAD) and specific caspase 3 inhibitor (DEVD) can still attenuate apoptosis, necrosis and caspase-3

activity after an extended period of reoxygenation (36 hrs) using the isolated adult rat ventricular myocytes model of ischemia and reperfusion.

5. To determine whether the administration of broad spectrum caspase inhibitor (ZVAD) and specific caspase 3 inhibitor (DEVD) at reperfusion can improve post ischemic functional recovery of isolated adult rat papillary muscles when using the work-loop technique to measure power output.

CHAPTER 3

Materials and Methods

3.1 Animals

Adult male Sprague-Dawley rats (350 ± 100 g body mass; mean ± SD) were used in these experiments. All animals were originally purchased from Charles River Laboratories (Wilmington, UK) then were kept and received human care at Coventry University Animal House (Coventry, UK). They were handled in compliance with the home office *Guidance on the Operation of Animals (Scientific Procedures) Act 1986.*

3.2 Chemicals and drugs

Chemicals used to prepare Krebs Henseleit buffer and triphenyltetrazolium chloride (TTC) used to stain the infarction were purchased from Sigma-Aldrich chemicals (Poole, UK). Cell permeable Caspase inhibitors were purchased from Calbiochem (Nottingham, UK). ZVAD-fmk (ZVAD) is a broad spectrum, non-selective and irreversible caspase inhibitor. Ac-DEVD-CMK (DEVD) is an irreversible inhibitor of caspase-3. Wortmannin (WORT) (Tocris, Bristol, UK) is a potent, selective, cell-permeable and irreversible inhibitor of phosphatidylinositol 3-Akt (PI3-Akt). Each inhibitor was dissolved in dimethylsulphoxide (DMSO) and aliquots were frozen at -20 ^{o}C. Aliquots were then diluted in Krebs Henseleit buffer immediately before use to a final concentration of 0.1-25μm for ZVAD, 0.07-25μm for DEVD (Mocanu *et al*., 2000, Kang *et al*., 2000) and 100nM for Wortmannin. The final concentrations of the DMSO in the final buffer solution did not exceed 0.02%, a

concentration which has previously been shown to have no effect on cardiac function or infarct size (Mocanu *et al*., 2000).

3.3 Krebs Henseleit Buffer (KHB)

Hearts were perfused with KHB, which mimics the ionic content of blood or plasma (Krebs & Henseleit, 1932). The KHB was composed of (in mM): NaCl 118.5, $NaHCO_3$ 25.0, KCL 4.8, $MgSO_4$ 1.2, KH_2PO_4, 1.2, $CaCl_2$ 1.7, and glucose 12. The buffer was prepared freshly each day and was continuously bubbled with 95% oxygen and 5% carbon dioxide. The temperature maintained at 37 ^oC and pH at 7.4-7.5.

3.4 Langendorff heart reperfusion studies

In the Langendorff perfusion studies, the heart aorta is cannulated and perfused in a retrograde fashion (Langendorff, 1895). In this mode the aortic valves are forced shut because of the weight of the perfusate column, and the perfusion fluid is then directed into the coronary arteries, thereby perfusing the entire ventricular mass of the heart.

3.4.1 Heart Isolation

Rats were killed by cervical dislocation, and then transferred to the dissecting table. The neck was immediately cut to relieve pressure on the heart via blood drainage from carotid arteries. The thoracic diaphragm was accessed through a trans-abdominal incision and was then dissected to expose the thoracic cavity. The anterior chest wall was then reflected and removed to expose the heart. The heart was then isolated by cutting the aorta, vena cava and pulmonary vessels. The heart was then immediately immersed into a dish containing

ice-cold KHB. Thereafter, the heart was quickly cannulated via an aortic cannula attached to the Langendorff perfusion system.

3.4.2 Langendorff perfusion system

In this perfusion system, the heart aorta was cannulated via an aortic cannula which was then attached to a reservoir containing oxygenated KHB (Figure 3.1) at a height of 0.8 m. The Langendorff perfusion system and the heart chamber are jacketed by warm water to maintain the temperature at 37 ^{o}C.

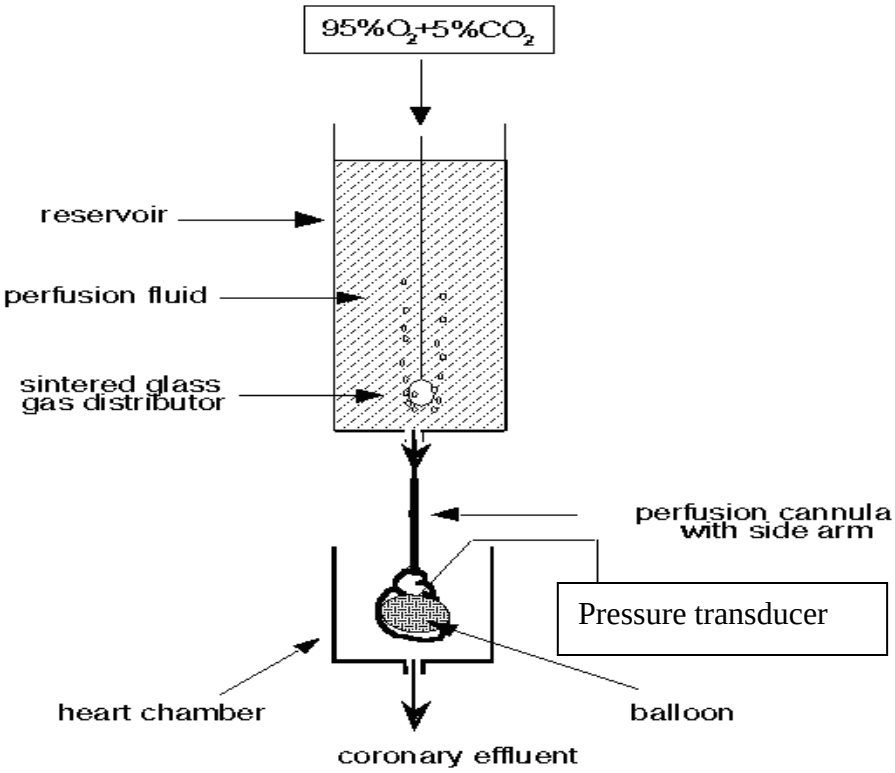


Figure 3.1: Langendorff perfusion system (adapted from Sutherland & hearse, 2000)

The heart was perfused retrogradely through the aorta using oxygenated KHB. The KHB was delivered down the aorta at a constant pressure of 80 mmHg. Thus, the aortic valve

forced closed and the perfusion fluid was directed into the coronary arteries and thereby infusing the entire ventricular mass and then drained into the right atrium via the coronary sinus.

Once the cannulation was completed, the left atrium was cut away and a latex balloon was inserted into the left ventricle. The balloon was inflated with water to give a preload of 4-8 mm Hg. The balloon was then attached to a physiological pressure transducer and a bridge Amp connected to a power lab (ADI Instruments, UK). Once the balloon was in position, left ventricular systolic developed pressure and heart rate were measured.

3.4.3 Measured haemodynamic parameters

Once the procedure of heart cannulation was completed, the coronary perfusion was started and contractile function of the heart was started within a few seconds. Heart rate (HR) and left ventricular developed pressure (LVDP) were measured using the latex balloon inserted into the left ventricle. Coronary flow (CF) was measured by collecting the perfusate at a regular time interval into a graduated cylinder. Temperature of the heart was continuously measured and maintained at 37 ^{o}C.

3.4.4 Inclusion/exclusion criteria

A set of exclusion criteria were used to ensure experimental reproducibility:

- LVDP at the end of stabilisation priod < 90 mmHg
- CF at the end of stabilisation period < 5 ml/minute or > 25 ml/minute
- HR at the end of stabilisation period < 290 beats /minute
- Failure to revert arrhythmia within 5 minutes.

3.4.5 Experimental protocol

Hearts were allowed to stabilize for 20 minutes before exposure to regional ischemia for 35 minutes. Regional ischemia was induced by occluding the left main coronary artery. This was done using a threaded surgical needle passed under the artery, with the ends of the thread passed through small plastic tubes to form a snare (Figure 3.2). Ischemia was induced by tightening the snare and was confirmed by a decrease in LVDP and coronary flow. After 35 minutes ischemia the snare was released and the heart was reperfused for 120 minutes.

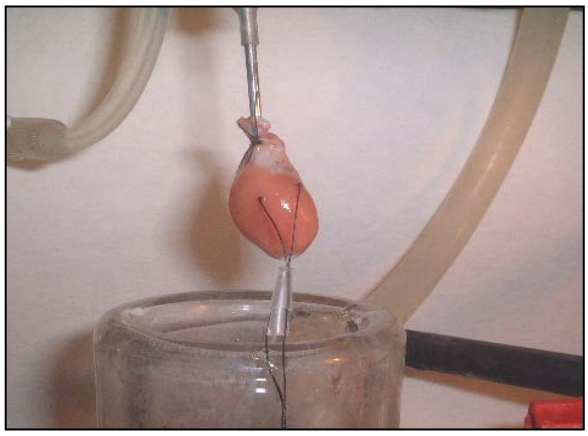


Figure 3.2: Photograph showing surgical thread passed under the left main coronary artery, with the ends of the thread passed through small plastic tubes to form a snare. Ischemia was induced by tightening the snare

The hearts were allocated to one of the experimental groups as shown in Figure 3.3 (n=5 each). Same ischemic control group was used in all langendorff experiments.

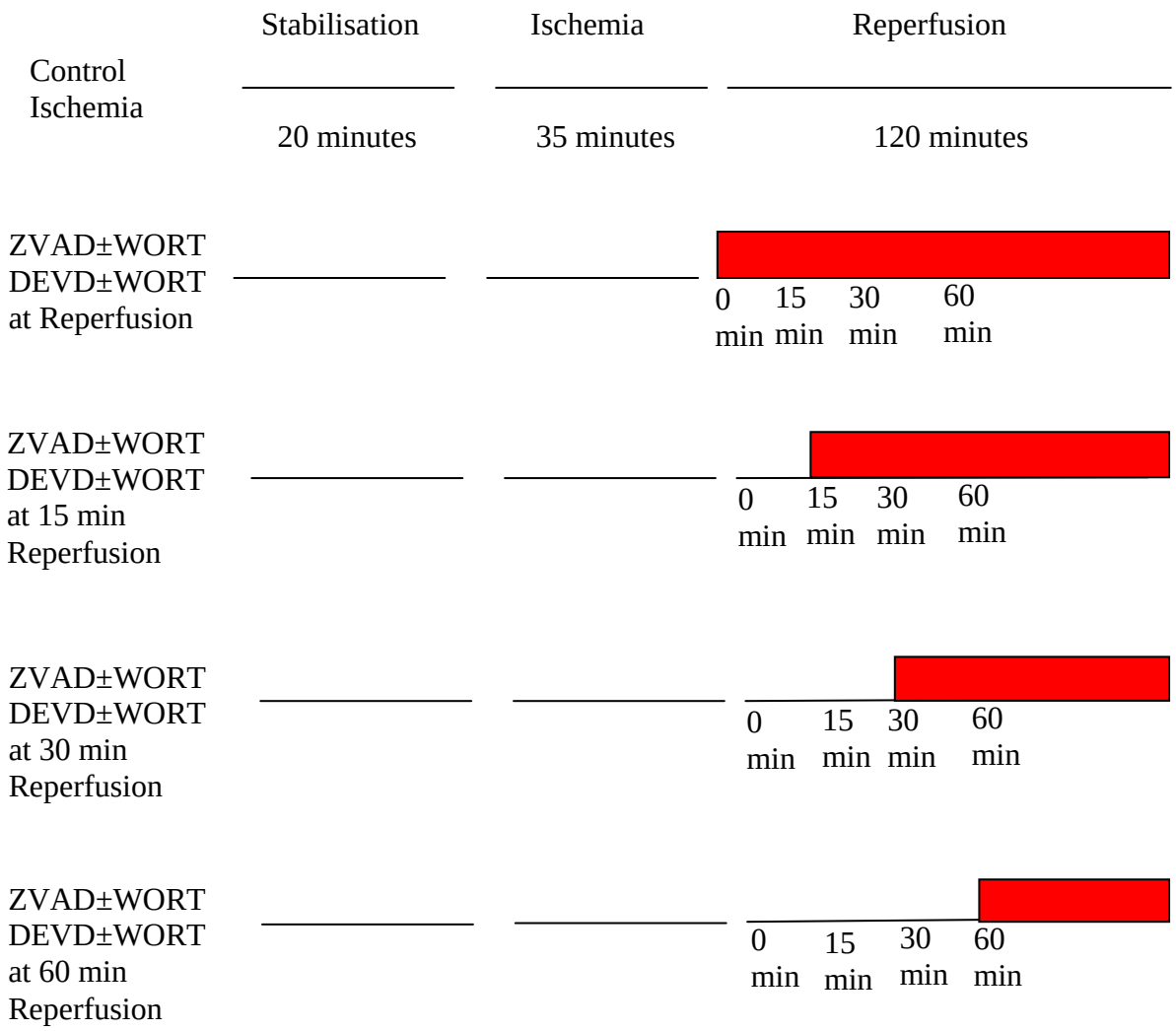


Figure 3.3: Illustrative diagram showing the experimental protocol. Control ischemic hearts were stabilised for 20 minutes before being exposed to 35 minutes regional ischemia, followed by 120 minutes perfusion with normal KHB. Treatment groups, hearts were treated with either broad spectrum caspase inhibitor (ZVAD, 0.1µM) or specific caspase-3 inhibitor (DEVD, 0.07µM) in the presence and absence of PI3-Akt specific inhibitor (WORT, 100nM). Treatments were introduced at different time points during reperfusion, at the start of reperfusion, 15 minutes, 30 minutes and 60 minutes after starting reperfusion. (n=5 each).

3.4.6 Infarct size measurement

At the end of the 120 minutes reperfusion period, the snare was tightened to re-occlude the coronary artery. Evans blue solution (0.25%) was infused slowly into the aorta to delineate the non-ischemic zone of the myocardium as a dark blue area (Figure 3.4). After freezing at -20 ^{0}C, hearts were sliced into 1 mm-thick transverse sections and incubated in triphenyltetrazolium chloride solution (1% phosphate buffer, pH 7.4) at 37 ^{0}C for 15 minutes. The sections were then fixed overnight in 10% formalin. At the end of this procedure, the risk area stained red or pink and the infarcted tissue appeared pale (Figure 3.5). The slices were then drawn onto acetate sheets. Computerized planimetry (ImageTool version 3.1, Rockford, USA) was used to measure the areas of infarcted tissues and risk area. The Infarction/Risk ratio (I/R %) was calculated as total infarcted tissue/total risk area x 100 for each heart.

Figure 3.4: Photograph showing infused Evans blue solution into the aorta to delineate the non-ischemic zone of the myocardium as a dark blue area and risk zone as pink area.

Fig 3.5: Heart slice showing the infarcted pale area and pinkish risk area after staining with triphenyltetrazolium chloride (TTC).

3.5 Western blot studies

3.5.1 Tissue Isolation

Adult male Sprague-Dawley rats (350 ± 100 g body mass; mean ± SD) hearts were isolated as described earlier (section 3.4.1), then quickly cannulated and perfused retrogradely with KHB using Langendorff perfusion system (section 3.4.2). At the end of reperfusion period, the snare was tightened to re-occlude the coronary artery. The hearts were then perfused with 0.25% Evans Blue via the aorta to delineate the non-ischemic zone of the myocardium which stained blue, and the risk zone, stained pink (Figure 3.4). Risk areas were separated from the heart using a sharp sterile scalpel, then frozen in liquid nitrogen and stored at -80°C.

3.5.2 Experimental groups

3.5.2.1 Cytochrome-c analysis

Hearts were allocated to one of the experimental groups showed in Figure 3.6.

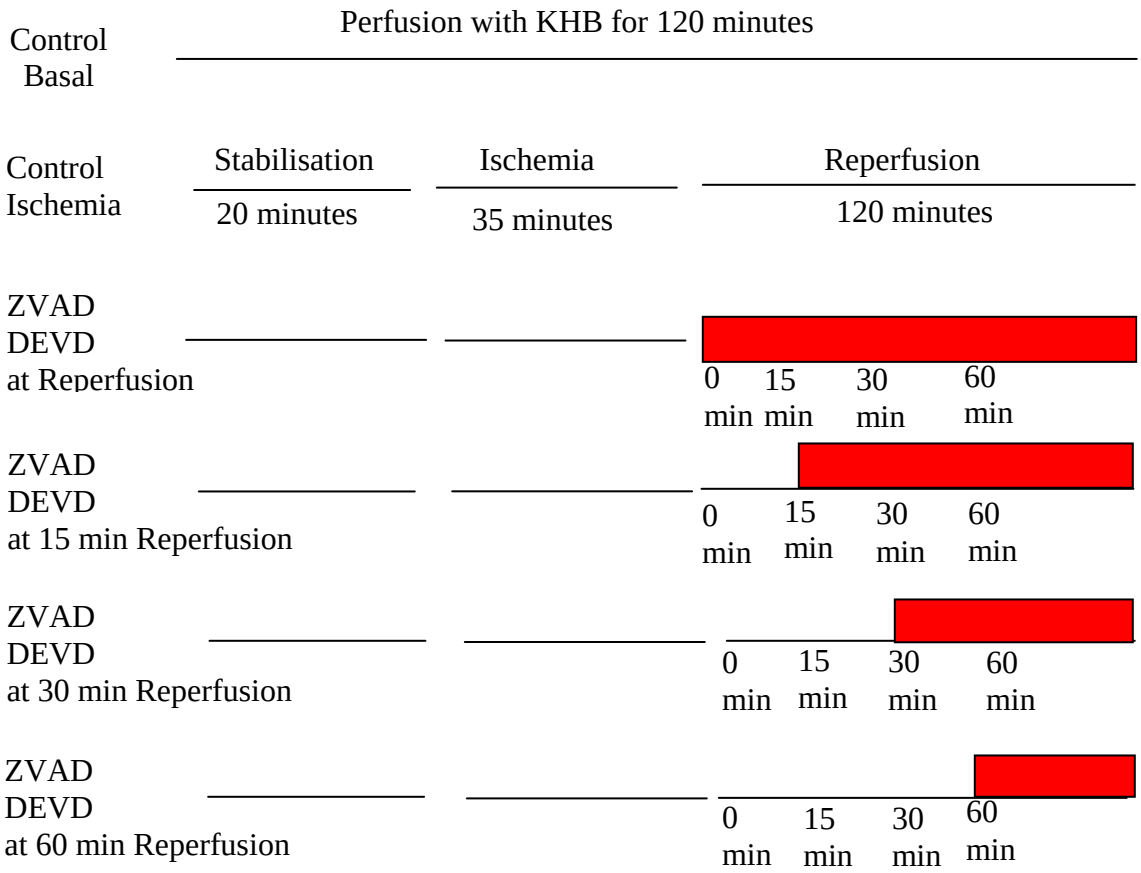


Figure 3.6: Illustrative diagram showing the experimental protocol for cytochrome-c analysis using western blot. Basal control hearts were perfused with normal KHB for 120 minutes. Control ischemic hearts were stabilised for 20 minutes before being exposed to 35 minutes regional ischemia, followed by 120 minutes perfusion with normal KHB. Treatment groups, hearts were treated with either broad spectrum caspase inhibitor (ZVAD, 0.1µM) or specific caspase-3 inhibitor (DEVD, 0.07µM). Treatments were introduced at different time points during reperfusion, at the start of reperfusion, 15 minutes, 30 minutes and 60 minutes after starting reperfusion. At the end of the experimental protocol, risk areas were separated from the heart using a sharp sterile scalpel, then frozen in liquid nitrogen and stored at -80°C for later western blot analysis. (n=5 each group). **Drug treatment**

3.5.2.2 Phospho-Akt analysis

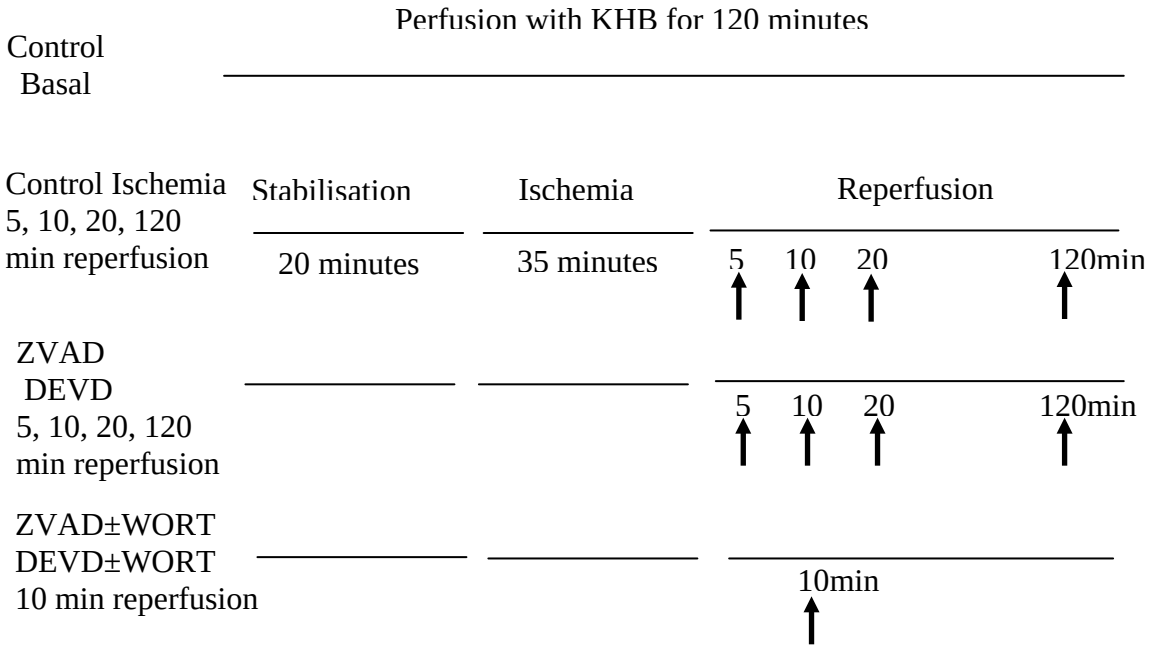


Figure 3.7: Illustrative diagram showing the experimental protocol for phosphor PI3-Akt analysis using western blot. Basal control hearts were perfused with normal KHB for 120 minutes before tissue isolation. Control ischemic hearts were stabilised for 20 minutes before being exposed to 35 minutes regional ischemia, followed by 5, 10, 20 or 120 minutes reperfusion with normal KHB before tissue isolation. Treatment groups, hearts were treated with either broad spectrum caspase inhibitor (ZVAD, 0.1µM) or specific caspase-3 inhibitor (DEVD, 0.07µM) for 5, 10, 20 or 120 minutes reperfusion before tissue isolation. PI3-Akt inhibitor (Wortmannin, WORT, 100nM) treated groups in the presence or absence of caspase inhibitors (ZVAD/0.1 µM, DEVD/0.07µM) perfused for 10 minutes before tissue isolation. ↑ Indicates time of tissue isolation (n=3 each group).

3.5.3 Mitochondrial and Cytosolic Protein Isolation

Mitochondrial and cytosolic proteins were isolated using a method previously described by Iwai *et al* (2002). Frozen tissue from the risk area was homogenized (Ultra-Turrax–T25 Basic ,IKA® - WERKE) in ice cold isolation buffer (in mM: Mannitol 210, Sucrose 70, EDTA 1, DTT 10, PMSF 0.5, Protease inhibitor cocktail 1.5 tab and Cyclosporine 0.1 µM, pH 7.4). Cyclosporine (Tocris, Bristol, UK) was included in the isolation buffer to reduce the permeability transition pore opening during protein isolation (Bouutaite *et al.,* 2002).

The homogenized tissue was then centrifuged (Jouan HS centrifuge, UK) at 900 x g for 10 min at 2 ^{0}C. The supernatant was then decanted into fresh pre-chilled tubes and then centrifuged at 8000 x g for 30 min at 2^{o}C.

The resulting supernatant was the cytosolic fraction, which was then decanted off and centrifuged at 100,000 x g (Sorvall ultra centrifuge, UK) for 30 min at 2^{o}C to pellet any mitochondrial proteins. 2 x 5 µl aliquots were taken for an assay of protein concentration (section 3.5.5). The cytosolic fraction was assayed for citrate syntahse (section 3.5.6). The remaining supernatant was then further diluted 2x in sample buffer (Tris-HCL 250 mM, SDS 4%, Glycerol 10%, Bromophenol blue 0.006%, ß-mercaptoethanol 2%, pH 6.8) and subsequently boiled for 10 min at 100^{o}C before storage at -20^{o}C for later electrophoresis (section 3.5.7).

The mitochondrial pellet was resusupended with ice cold isolation buffer (in Mm, Mannitol 210, Sucrose 70, TRIS 10, pH 7.4). The mitochondrial pellet was broken down by multiple freeze thaw cycles using liquid nitrogen. 2 x 5 µl aliquots were taken for protein concentration assay (section 3.5.5). The remaining solution was diluted with 2 x volume sample buffer and stored at -20^{o}C for later electrophoresis (section 3.5.7).

3.5.4 Protein isolation for phospho Akt analysis

The frozen risk area tissue was homogenized (Ultra-Turrax–T25 Basic ,IKA®-WERKE) in ice-cold lysis buffer (in mM NACL 100, TRIS 10, EDTA 1, (pH 8.0), Sodium Pyrophosphate 2, Sodium Floride 2, ß-Glycerophosphate 2, PMSF 0.1 mg/ml and protease inhibitor cocktail tablets). The homogenised sample was then centrifuged (Jouan HS centrifuge, UK) at 11,000 rpm, and 4°C for 10 minutes. 2 x 5 µl aliquots were taken for protein concentration assay (section 3.5.5). The remaining supernatant was then further diluted in 2x volume sample buffer and subsequently boiled for 10 min at 100^{o}C before storage at -20^{o}C for later electrophoresis (section 3.5.7).

3.5.5 Sample protein concentration determination

The bicinchoninic acid (BCA ™) protein assay kit (Perbio Science**,** Cramlington, UK) was used to determine sample protein concentration. The principle of the assay relies on the formation of a Cu^{2+}-protein complex under alkaline conditions, followed by reduction of the Cu^{2+} to Cu^{1+} as shown below:

Protein (peptide bonds) + Cu^{2+} → tetradentate-Cu^{1+} complex

Cu^{1+} + 2 bicinchoninic acid (BCA) → BCA-Cu^{1+} complex

The amount of reduction is proportional to the protein present. BCA forms a purple-blue complex with Cu^{1+} in alkaline environments, which can be measured by optical densitometry at 562 nm (Smith *et al*., 1985). The relationship between the absorbance at 562 nm and protein content is linear over a wide concentration range (20-2000µg/ml); therefore the protein content of the samples can be estimated through comparison with a standard curve.

A standard curve was generated using bovine serum albumin (BSA) as a standard protein. BSA at incremental concentration was mixed with suspension buffer, BCA buffer and water as shown in table 3.1.

Sample	**Final protein concentration (µg)**	**BSA (µl) from 2 mg/ml stock**	**Suspension buffer (µl)**	**Distilled water (µl)**
A	**0**	**0**	**5**	**45**
B	**20**	**10**	**5**	**35**
C	**40**	**20**	**5**	**25**
D	**60**	**30**	**5**	**15**
E	**80**	**40**	**5**	**5**

Table 3.1: Table showing the volumes used to prepare the calibration curve using BSA standards.

Then BSA standards samples were incubated at 37^{o}C for 30 minutes. The absorbance at 562 nm was then measured using spectrophotometer (Shimadzu UV 2100, UK). A standard curve was produced (Figure 3.8).

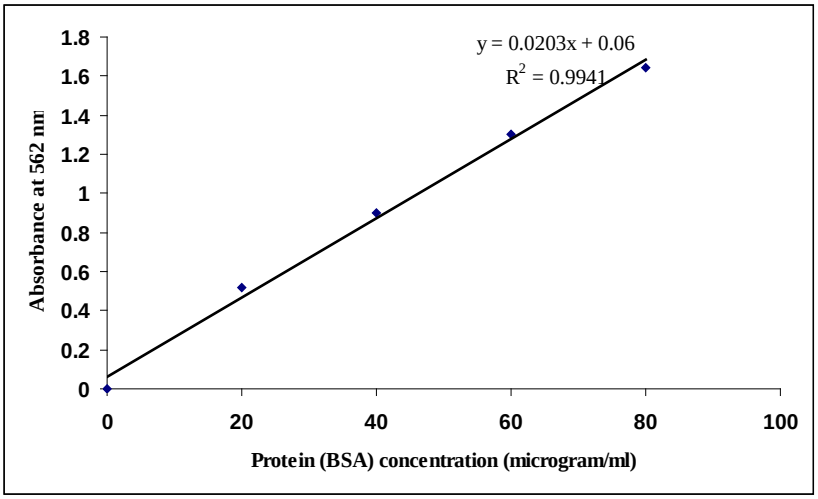


Figure 3.8: Calibration curve produced with BSA as standard protein.

The protein concentrations of cytosolic and mitochondrial samples were then quantified using the same method, and the sample absorbance value compared against the standard curve to provide an estimate of the protein concentration. This protein estimation method enabled equal loading of protein into polyacrylamide gel (section 3.5.7).

3.5.6 Citrate synthase assay

Citrate synthase is a mitochondrial matrix enzyme, which can be considered as a mitochondrial marker. This assay was done to assess any damage to mitochondria during protein isolation. Citrate synthase is the initial enzyme of the tricarboxylic acid (TCA) cycle) (Srere, 1969). This enzyme catalyzes the reaction between acetyl coenzyme A (acetyl-CoA) and oxaloacetic acid (OAA) from citric acid. The acetyl-CoA contributes 2 carbons to the 4 carbons of oxaloacetate resulting in citrate with 6 carbons. The hydrolysis

of the thioester of acetyl-CoA results in the formation of CoA with a thiol group (CoA-SH) (equation 1).

Equation 1: Reaction catalyzed by citrate synthase

Acetyl CoA + Oxaloacetate → Citrate + CoA-SH + H^+ + H2O

The thiol reacts with the 5,5'-dithiobis-(2-nitrobenzoic acid; DTNB) in the reaction mixture to form 5-thio-2-nitrobenzoic acid (TNB). This yellow product (TNB) is observed spectrophotometrically by measuring absorbance at 412 nm (equation 2).

Equation 2: Colorimetric reaction in assay:

CoA-SH + DTNB → TNB + CoA-S-S-TNB.

The assay solutions contained in mM (Tris 100 (pH 8.1), OAA 10, Acetyl CoA 30, DTNB 10; Sigma-Aldrich, Poole, UK) and was warmed to 25 ^oC. Cytosolic samples reactions were prepared in duplicate 1 ml cuvettes (Table 3.2).

Sample	**100 mM Tris buffer**	**30 mM AcetylCoA solution**	**10mM DTNB solution**	**10mM OAA solution (To be added last)**
50 µl	**880 µl**	**10 µl**	**10 µl**	**50 µl**

Table 3.2: Table showing the volumes used to prepare the citrate synthase assay.

The absorbance of the reaction was followed using a spectrophotometer (412nm) at kinetic mode (Perkin Elmer, Bucks, UK) for 1.5 minute to measure the baseline reaction (the endogenous levels of thiol or deacetylase activity). Then 50 µl of OAA was added to initiate the reaction and the absorbance was again followed for 1.5 min to measure the total

activity. The absorbance values (A 412) were plotted against time for each reaction. The change in absorbance was measured (ΔA412)/minute, in the linear range of the plot, for the endogenous activity and for the total activity after addition of OAA. The net citrate synthase activity was then calculated by subtracting the (ΔA412)/minute of the endogenous activity from the (ΔA412)/minute of the total activity of the sample. Finally this value used for calculating the citrate synthase activity (equation 3)using the following equation:

Equation 3: Citrate Synthase Activity (μmole/ml/min) = $\frac{(\Delta A412)/min \times V(ml) \times dil}{\varepsilon^{mM} \times L\ (cm) \times V_{enz}\ (ml)}$

Where dil= the dilution factor of the original sample; V(ml)=the reaction volume=1ml; Venz (ml)= the volume of the enzyme sample in ml; ε^{mM} (mM^{-1} cm^{-1})= the extinction coefficient of TNB at 412 nm which is 13.6; and L (cm)= pathlength for absorbance measurement, for 1 ml cuvette, pathlength = 1 cm.

3.5.7 Polyacrylamide Gel Electrophoresis (PAGE)

3.5.7.1 Gel preparation

Each 12.5 % acrylamide gels was prepared by adding 12ml distilled water, 15ml 30 % acrylamide (Bio-Rad, Hemel Hempstead, UK), 15 ml running gel base, 40μl TEMED (Bio-Rad, Hemel Hempstead, UK) and 200μl ammonium persulphate (APS, Fisher Scientific, Loughborough, UK). The gel was made between two glass plates separated by spacers of 1.5 mm thickness and then secured in a casting frame. Once the gel was set, a 5% stacking gel (7 ml distilled water, 3 ml stacking gel base, 2 ml 30% acrylamide, 24μl TEMED and

120 µl 10% ammonium persulfate) was added at the top. Protein loading wells were made in the stacking gel by inserting a multi well comb.

3.5.7.2 Electrophoresis

The gel was then placed into a tank filled with a 1X running buffer. A total of 50-60 µg of protein for each sample was loaded into the gel. Precision plus molecular marker (New England Biolabs, Hitchin, UK) and biotinylated ladder (New England Biolabs, Hitchin, UK) (10 µl each) were also loaded. The gel was then allowed to run for 55 min at 120 V using the powerpac HC ^{TM} (Biorad, Hemel Hempstead, UK).

3.5.7.3 Electrotransfer

Following electrophoresis, the gel was removed and immersed in 1X transfer buffer and allowed to equilibrate for 15 minutes under gentle agitation. The Hybond-P Polyvinyl Difluoride membrane (PVDF) (Amersham, Buckinghamshire, UK) was cut to the size of the gel and pre-wetted with 100% methanol for 10 seconds and then washed out with distilled water and then immersed in 1X transfer buffer for 15 minutes. Sponges and the Whatman filter papers were also wetted with transfer buffer.

The gel was carefully opposed to the PVDF membrane, and then sandwiched between sponges and Whatman filter papers, with care taken to avoid air bubbles. The transfer cassette was then placed in a transfer tank and immersed with transfer buffer and allowed to transfer for 2 hours at 50 V.

After transfer, the membrane was removed and stained with ponceau stain (Sigma-Aldrich, Poole, UK) to assess efficient transfer. Then the stain was washed out with TBS-Tween buffer.

3.5.7.4 Membrane Blocking

The membrane was blocked with 5% milk (Marvel) buffer (25 ml TBS-Tween and 1.25g Marvel milk powder), for 1 hour with gentle agitation in an orbital shaker at room temperature.

3.5.7.5 Immunoblotting

After blocking, the membrane was washed 3 times with TBS-Tween for 5 minutes each. The membrane was then incubated with the primary antibody diluted in antibody dilution buffer (0.1 % milk in TBS-T for cytochrome c and VDAC; 5% BSA in TBS-T for phospho-Akt) overnight with gentle agitation in an orbital shaker at 4^{o}C. Cytochrome c primary antibody (New England Biolabs, Hitchin UK), VDAC (abcam, Cambridge, UK) and phospho-Akt (Ser473) (New England Biolabs, Hitchin, UK) were used in 1:1000 dilutions.

The membrane was then washed with TBS-Tween buffer for 3 x 5 min. The membrane was then incubated with HRP conjugated secondary antibody buffer (anti-rabbit IgG antibody, New England Biolabs, Hitchin, UK) at a dilution of 1:2000 for 1 hour with gentle agitation at room temperature. The membrane was then washed again with TBS-Tween buffer for 3 x 5 minutes.

3.5.7.6 Protein detection

Proteins were detected using enhanced chemiluminescence (ECL PLUS, Amersham, Buckinghamshire, UK). Its principle relies on the enzymatic conversion of a luminol-like molecule to a reactive molecule by horseradish peroxidase (HRP). This molecule generates light in the presence of hydrogen peroxide (Thorpe *et al*., 1986).

The membrane was incubated with ECL PLUS reagents for 5 minutes, then wrapped in a piece of Clingfilm and then exposed to radiography for one minute. The radiography film (Amersham, Buckinghamshire, UK) was then placed in a developer and then fixer solutions (Kodack, UK), then washed with distilled water and allowed to dry.

3.5.7.7 Detection of loading control (α-tubulin)

The PVDF membranes were reprobed with the loading control α-tubulin as described by Liao *et al*. (2000). After incubation with the ECL Plus Western Blotting detection kit, the PVDF membranes were kept in fresh TBS-T at 4 ^{o}C for a day. The PVDF membranes were washed 3 times for 5 minutes with TBS-T. These membranes were reprobed with α-tubulin (Abcam, Cambridge, UK) (dilution 1:2000) in 5% milk overnight at 4^{o}C.

Membranes were then incubated with the secondary antibody at 1:5000 dilutions (Goat polyclonal to Rabbit IgG HRP, abcam, Cambridge, UK). After washing, protein was detected as described in section 3.5.7.6 above but exposed to radiography for only 10 seconds.

3.5.7.8 Densitometry

Autoradiography films were scanned on a scanner and the digital image was then imported to Quantity One Analysis Software (BioRad, Hemel Hempstead, UK). The relative densitometry of each band was determined after subtracting the background noise.

3.6 Adult rat ventricular myocytes isolation

3.6.1 Cardiomyocytes Isolation

Adult rat ventricular myocytes were isolated from Sprague Dawley male rats (300 ± 100g body mass) by enzymatic dissociation method. The rats were killed by cervical dislocation, then the hearts were quickly excised and mounted on a modified Langendorff apparatus and perfused for 5 minutes with perfusion buffer (PB) containing (in mM); NaCl 116, KCl 5.4, $MgSO_4.7.H_20$ 0.4, Glucose 10, Taurine 20, Pyruvate 5, $Na_2HPO_4.12H_20$ 0.9, $CaCl_2$ 1.7 and 25 NaHCO3. The PB was bubbled with 95% O_2 and 5% CO_2, pH maintained at 7.4 and temperature at 37^{o}C. Then the hearts were switched to calcium free PB for another 5 minutes. Finally hearts were perfused with PB containing 0.75mg/ml Collagenase (Worthington type II) and 44μM calcium for 5-10 min at a rate of 10ml/min. The hearts were then removed from the perfusion apparatus and the atria trimmed away. The ventricles were minced and incubated in a shaking bath for 10-20 min in collagenase-containing PB and bubbled with 95% O_2 and 5% CO_2. Cells were then filtered through nylon mesh and washed with restoration buffer (in mM); 116 NaCL, 5.4 KCl, 0.4 $MgSO_4$, 10 Glucose, 20 Taurine, 5 Pyruvate, 0.9 $NaHPO_4$, 25 $NaHCO_3$, 2 Carnitine, 5 Creatine, 50μM $CaCl_2$ 2g

BSA and 1% pen-strep (pH 7.4, 37^{o}C). The calcium concentration of the cell suspension was gradually increased to a final concentration of 1.25 mM. The cells were kept in incubator at 37^{o}C and an atmosphere of 21% O_2 and 5% CO_2. For each experiment, a different heart was used to ensure that variability between preparations was not artificially reduced.

3.6.2 Experimental protocol

The hypoxic condition was created by replacing the normal restoration buffer with ischemic buffer (containing in mM; 137 NaCl, 12 KCl, 0.49 $MgCl_2$, 0.9 $CaCl_2$, 4 HEPES, 20 Na lactate, 10 deoxyglucose) and then the cells were incubated in an air-tight hypoxic chamber with an atmosphere of 5% CO_2 and 95% N_2 at 37^{o}C. For reoxygenation, cells were removed from the hypoxic chamber and the hypoxic medium was replaced with the normal restoration buffer. The cells were then placed in an incubator at 37^{o}C and atmosphere of 21% O_2 and 5% CO_2. Experimental groups were divided into control groups, cells were exposed to 6 hrs of hypoxic condition and 18 hours of reoxygenation condition. Treated groups, cells were exposed to 6 hrs of ischemic conditions and 18 hrs of reoxygenation condition where caspase inhibitors (ZVAD-fmk, 25µM and Ac-DEVD-CMK, 25µM) were added at the start of roxygenation, 15 min, 30 min and 60 min after starting reoxygenation in the presence or absence of PI3-Akt inhibitor; wortmannin (WORT, 100 nM) Figure 3.9. The same control hypox/reox group was used in all experimental studies using adult rat ventricular myocytes model.

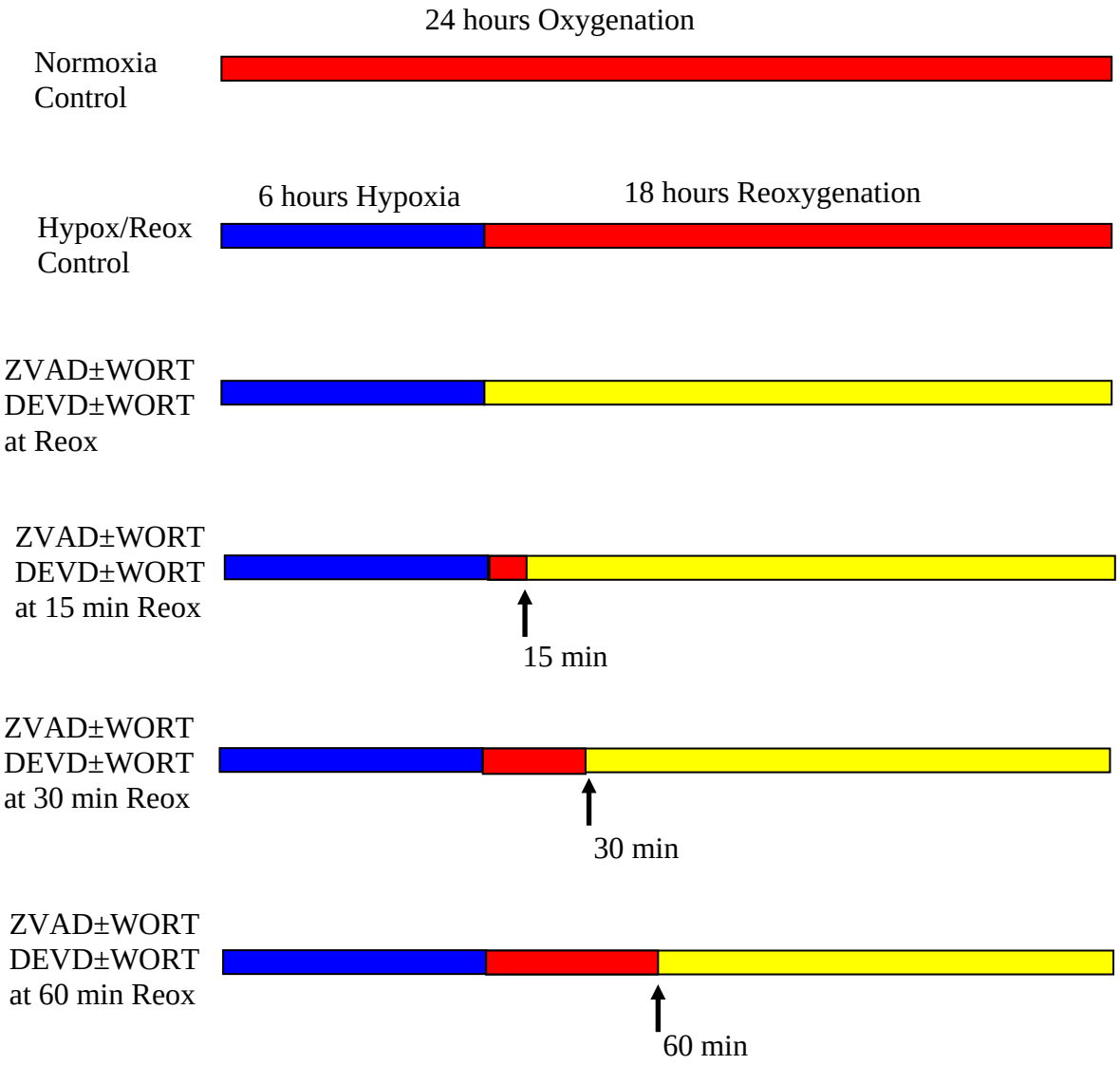


Figure 3.9: Illustrative diagram showing the experimental protocol for isolated adult rat ventricular myocytes. Normoxia contol group, cells were exposed to normal oxygenated conditions for 24 hours. Hypox/Reox control group cells were exposed to 6 hrs of hypoxic conditions and 18 hours of reoxygenation condition. Treated groups, cells were exposed to 6 hrs of ischemic conditions and 18 hrs of reoxygenation condition where caspase inhibitors (ZVAD, 25µM and DEVD, 25µM) were added at the start of roxygenation, 15 min, 30 min and 60 min after starting reoxygenation in the presence or absence of PI3-Akt inhibitor; wortmannin (WORT, 100 nM). (n=8).

Hypoxia **Oxygenation** **Drug treatment**

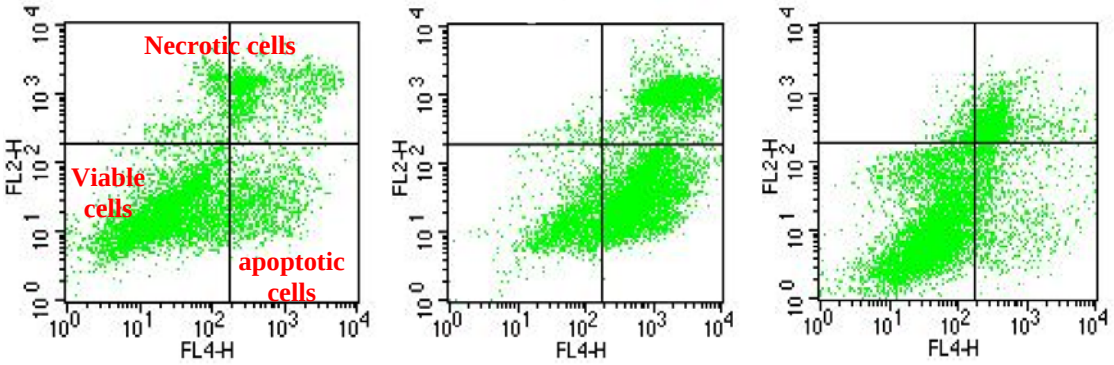
For prolonged reperfusion studies, the same experimental protocol (figure 3.9) was followed, however at the end of the first 18 hours reperfusion, cells were collected and then resuspended in a fresh reperfusion buffer and caspase inhibitors added freshly. Then they were incubated for another 18 hours in the same reoxygenation conditions to make 36 hours total reoxygenation time.

3.6.3 Quantitative Analysis of Cellular Viability, Apoptosis and Necrosis

The Vybrant ® Apoptosis Assay Kit #10 (Invitrogen, Paisley, UK) was used for quantitative analysis of cellular viability, apoptosis and necrosis. This kit provides a rapid and convenient assay for apoptosis. In normal cells the distribution of phospholipids is asymmetric with the inner membrane containing anionic phospholipids, such as phosphotidyl serine (PS) and the outer membrane having mostly neutral phospholipids. In apoptotic cells, the amount of PS on the outer surface of the membrane increases exposing PS to the surrounding liquid (Martin *et al*., 1995). Annexin V, a calcium dependent phospholipids binding protein, has a high affinity for PS. Annexin V binds to PS in the outer membrane of apoptotic cells, thus providing a suitable way of detecting apoptosis (Martin *et al*., 1995). This kit contains, recombinant annexin V conjugated to allophycocyanin (APC) to stain apoptotoic cells. APC is an extremely fluorescent phycobiliprotein, which can be easily excited with a helium-neon (HeNe) laser at 633 nm. This kit also contains C12-resazurin which is reduced by viable cells to orange-fluorescent C12-resorufin Resazurin has been used extensively to detect the metabolic activity of many different cell types. Necrotic cells detected with the SYTOX ® Green dye which is

impermeant to live and early apoptotic cells, but stains dead cells with intense green fluorescence by binding to cellular nucleic acids.

At the end of the experimental protocol, the cells were harvested and transferred to a labelled ependorff tubes and centrifuged at 500 rpm for 5 mins. The supernatant was removed and the cell pellet washed with Annexin V buffer. Then the tubes were centrifuged again at 500rpm for 5 min. The supernatant was decanted off and the cells were suspended in Annexin V buffer. The cells were then stained with Annexin V conjugated APC, C12 resazurin and SYTOX Green dyes according to the kit instruction sheet. The ependorff tubes were then covered in foil to protect from light and incubated at 37^{o}C, 5% CO_2 for 15 min. The samples were then diluted with more Annexin V binding buffer and analysed immediately by flow cytometery (FACS, Becton Dickinson, Oxford, UK). The samples were analysed on the FL-2 and FL-4 channels and setup to count 10,000 events. The cells population should separate into three groups: live cells with only a low level of green and far-red fluorescence and a high level of orange fluorescence; apoptotic cells with a high level of far-red fluorescence, intermediate orange fluorescence, and no green fluorescence; and dead cells with a high level of green and far-red fluorescence and a low level of orange fluorescence. The most commonly used analysis plot consists of a square divided into four quadrants. The position of a dot on the plot depends upon whether the cell is positive for one flourchrome, both, or neither (Figure 4.6). The percentage of events that fall into each quadrant is reported by the computer, and this report correlates with the density of the dots in the respective quadrant. Numerous studies have shown the use of flow cytometry quadrant based analysis as a reliable method for the assessment of apoptotic rat ventricular myocytes (Vermis *et al*., 1995, vermis *et al*., 2000, Maddock *et al*., 2003).



a. Normoxia b. Hypox/Reox c. Anti-apoptotic drug (Caspase inhibitors)

Figure 3.10: Representative diagram showing flow cytometry analysis of adult rat ventricular myocytes using the Vybrant® Apoptosis Assay Kit #10. Myocytes were incubated with the reagents in the Vybrant® Apoptosis Assay Kit #10, and then analyzed by FACS flow cytometry. The SYTOX® Green fluorescence versus APC (allophycocyanin) fluorescence dot plot shows resolution of live, apoptotic, and necrotic cell populations. a. Normoxia, b. 6 hours of hypoxia followed by 18 hours of reoxygenation, c. anti-apoptotic drugs (caspase inhibitors).

3.6.4 Measurement of Caspase-3 activity

At the end of the experimental protocol, cells were harvested into labelled ependorff tubes, and then were centrifuged at 1200 rpm for 2min. Cells were then resuspended in Phosphate Buffer Saline (PBS). Cells were then fixed with 3% formaldehyde for 10 min at 37^{o}C. Cells were then permeabilized by adding ice cold 90% methanol for 30 min at 4^{o}C. Cells were then washed with incubation buffer (0.5% BSA in PBS) and then blocked with the incubation buffer for 10 min, and then blocked with the primary antibody (cleaved caspase-3 (Asp 175) (5A1) rabbit mAb; New England Biolabs, Hitchin, UK) at 1:100 dilution for 1 hour at room temperature. Cells were then washed using incubation buffer and then resuspended in alexa flour@ conjugated secondary antibody (Alexa flour@488 F (ab') 2 fragment of goat anti-rabbit igG, Invitrogen, Paisley, UK) at 1:1000 dilution for 30 min at

room temperature. Finally cells were washed with incubation buffer and resuspended in PBS and immediately analysed using flow cytometry on the FL1 channel (Vermes *et al*., 2000). Histograms were plotted for each of the groups showing the Mean fluorescence for 10,000 counts (Figure 4.7).

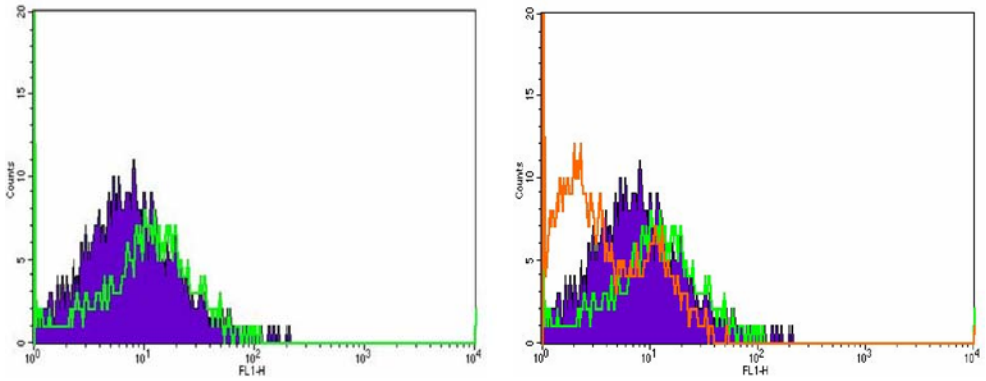


Figure 3.11: Representative flow cytometric histograms of intracellular Caspase-3 activity in myocytes subjected to normoxia conditions (blue), hypoxia/reoxygenation conditions (green) and hypoxia/reoxygenations conditions with caspase inhibitors treatment (red).

3.7 Work loop studies

3.7.1 Muscle preparation

Adult male Sprague-Dawley rats (250 g $\pm$ 100 g body mass) were used. 24 rats were obtained from Coventry University Animal House (Coventry, UK). All rats were killed by cervical dislocation in accordance with the home office *Guidance on the Operation of Animals (Scientific Procedures) Act 1986*. The heart was rapidly excised and pinned out on Petri dish on a Sylgard 184 base (Dow Corning) and immersed in fresh oxygenated Krebs Henseleit (KHB) buffer (in mM: NaCl 118.5, $NaHCO_3$ 25.0, KCl 4.8, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 1.7, and glucose 12, pH 7.4 at 20^{o}C) at room temperature.

An incision was made through the right ventricle to expose the papillary muscles. A suitable papillary muscle was then dissected by cutting through the valvular chordae tendeneae at one end and the ventricular muscle wall at the other end. Aluminium foil clips were wrapped as close as possible to the ends of the papillary muscle preparation around the tendon at one end and the ventricular wall at the other end. The dissection was performed rapidly at room temperature and KH buffer was continuously replaced with fresh oxygenated KHB.

The muscle was then attached horizontally via the foil clips to a force transducer (UFI, Pioden Controls Ltd, UK) at one end and a displacement transducer (V201, Ling Dynamic Systems, UK) at the other end in a flow through chamber containing circulating oxygenated (95% O_2; 5% CO_2) KHB maintained at 37^{o}C. A linear variable-displacement transducer (model DFG5.0, Solarton Metrology, UK) was used for position detection. Two platinum wire electrodes positioned parallel to the muscle within the chamber were used to stimulate the muscle preparation. The muscle was allowed 20 minutes before starting the experiment to allow both recovery from the dissection and adjustment to higher temperature of 37 ^{o}C.

3.7.2 Isometric studies

The muscle preparation was stimulated via the platinum electrodes while held at constant length to yield an isometric twitch, which was displayed and measured on a digital oscilloscope. Stimulation amplitude and muscle length were optimised via several isometric twitches to maximise force output. The muscle length was increased gradually in 0.2mm increments using a micromanipulator, and then active force was elicited and measured at

each length. This procedure was repeated until active force was seen to decline. The muscle length that generated the maximum active force was then measured using a graticule eye piece. The muscle length that generated the maximum active force under isometric conditions was defined as L_{max}.

3.7.3 Work loop studies

The work loop technique was used to measure the power output of the muscle during cyclical length changes (Josephson, 1985). The muscle was subjected to a sinusoidal length change wave form and phasically stimulated to simulate *in-vivo* function (Semafuko & Bowie, 1975).

Preliminary experiments were done to determine the optimal muscle length ($L_{opt,}$ the length for maximum work production), cycle frequency (frequency of the sinusoidal length changes), strain (extent of length change expressed as a proportion of L_{opt}) and phase shift (start of stimulation expressed relative to the maximum length achieved during the sinusoidal length change) that produce maximal power output. These optimal parameters were then used in all subsequent experiments.

In subsequent experiments , each muscle at L_{max} was subjected every five minutes to a set of four sinusoidal length change cycles at 6 Hz cycle frequency, -20 ms phase shift and strain of 0.12 for the whole experimental protocol. Muscle length and stimulation were controlled using custom written software (Testpoint, CEC, MA, USA). A plot of force against length produced a ‘work loop’ that represents the net work done by the muscle

during the length change cycle. The first work loop of each set of cycles was found to produce the maximum power output and was used to indicate power output of the muscle.

3.7.4 Experimental protocol

Ischemia was simulated by perfusion with hypoxic buffer (composition in mM: 118.5 NaCl, 25 $NaHCO_3$, 4.8 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 1.7 $CaCl_2$, and 7.0 choline chloride) bubbled with a gas mixture of 95% N_2: 5% CO_2. For reoxygenation, the hypoxic buffer was replaced with normal oxygenated (95% O_2:5% CO_2) KHB.

The experimental groups were baseline non-ischemic, ischemic control and treatment groups (Figure 3.12). In the baseline group, muscles were bathed continuously with circulating oxygenated normal KHB for 155 minutes. In the ischemic control group (n=6), muscles were bathed with circulating oxygenated normal KHB for 20 minutes, then bathed with circulating hypoxic buffer for 35 minutes, then reoxygenated with circulating normal oxygenated KHB for 100 minutes. Treatment groups (n=6 each), caspase inhibitors (ZVAD, 0.01µM or 2.5µM and DEVD, 2.5µM) were added at the start of reoxygenation (Figure 3.12). In each experimental group, four work loops were delivered every 5 minutes to monitor the power output of the muscle throughout each experiment.

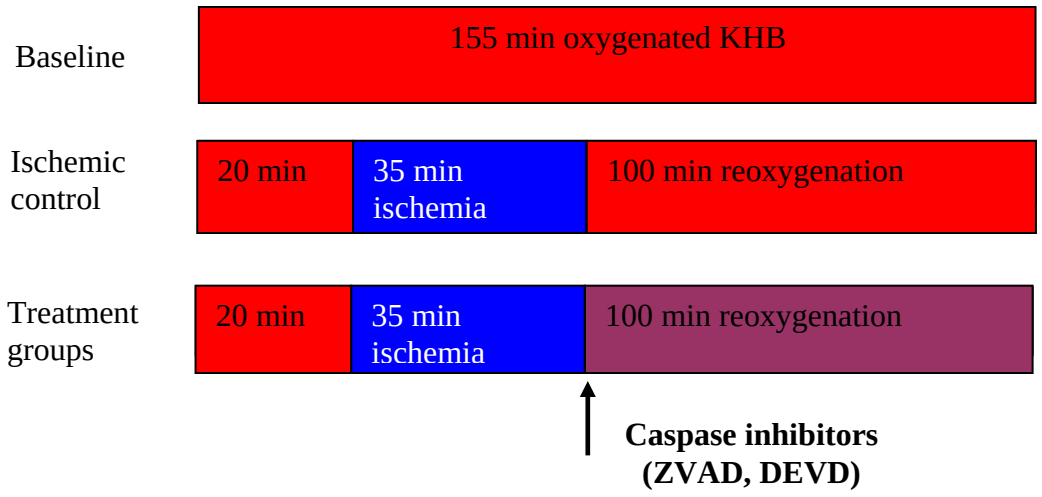


Figure 3.12: Illustrative diagram showing the experimental protocol used for work loop studies. Baseline group, muscles were bathed continuously oxygenated KHB for 155 minutes. Ischemic control group, muscles were bathed with oxygenated KHB for 20 minutes, then bathed with hypoxic buffer for 35 minutes, then reoxygenated with oxygenated KHB for 100 minutes. Treatment groups, caspase inhibitors (ZVAD, 0.01µM or 2.5µM and DEVD 2.5µM) were added at the start of reoxygenation (n=6 each). Normal oxygenated KHB Hypoxic buffer Drug treatment

3.7.5 Muscle mass measurement and dimension calculations

At the end of the experimental protocol, the papillary muscle was removed and dissected away from the tendon and the ventricular wall. Excess KH buffer was removed from the muscle before measurement of muscle mass. Muscle power output was then normalised to muscle mass (W Kg^{-1}). Mean muscle cross sectional area was calculated from muscle mass, muscle length and an assumed muscle density of 1060 kg m^{-3}. Isometric stress was then calculated as force divided by cross sectional area (KN m^{-2}).

3. 8 Statistical analysis

Results are presented as group means ± standard error of the mean (SEM). All data were analysed using SPSS statistical software (SPSS, version 14).

All values for I/R % are expressed as mean I/R % $\pm$ Standard Error of the Mean (SEM). Data were analysed using SPSS one-way ANOVA. Tukey's post hoc test was performed to test for significant differences between groups when a significant main effect (ANOVA) had been found. A p value of <0.05 was considered to be statistically significant.

All values for measured haemodynamic parameters (LVDP, HR, and CF) are expressed as mean stabilisation percentage $\pm$ Standard Error of the Mean (SEM). Data were analysed using two way ANOVA (time and experimental group) with Tukey's post hoc test to test statistical difference. One way ANOVA with Tukey's post hoc test was then used to test for statistical difference at specific time points (start of ischemia, end of ischemia, start of reperfusion and end of reperfusion period). A p value of <0.05 was considered to be statistically significant.

All values for densitometry values of western blot are expressed as mean density $\pm$ Standard Error of the Mean (SEM). Data were analysed using SPSS one-way ANOVA. LSD test was performed to test for significant differences between groups when a significant main effect (ANOVA) had been found. A p value of <0.05 was considered to be statistically significant.

All values of adult rat venricular myocytes studies were presented as mean % cell populations ± Standard Error of the Mean (SEM). Data was analysed using SPSS one-way ANOVA followed by Tukey's post hoc test to test for statistical difference. A p value of <0.05 considered to be statistically significant.

All data of maximum power output were expressed as a percentage of the mean of the first 20 minutes stabilisation values. All the percentage data were then transformed to arcsine values to meet the requirements of parametric statistical analysis. Using SPSS statistical software, two way ANOVA (time and experimental groups) with Tukey's post hoc test was used to test for statistical difference. One way ANOVA with Tukey's post hoc test was then used to test for statistical difference at specific time points. One way ANOVA with Tukey's post hoc test was used to test for significant differences in isometric stress and mean stabilisation power output between experimental groups. Significance being attributed at $p<0.05$.

CHAPTER 4

Myocardial Ischemia Reperfusion Injury Is Reduced By Administration Of Caspase Inhibitors At Various Time Points During Reperfusion.

4.1 Introduction and Objectives

Coronary heart diseases and subsequent myocardial infarction is the leading cause of high mortality and morbidity worldwide (Okrainec *et al*., 2004). The prerequisite for rescuing viable myocardium after acute myocardial ischemia is the early restoration of coronary blood flow (reperfusion) (de Boer *et al*., 1994) However, reperfusion itself has the potential to introduce additional lethal injury that is not evident at the end of ischemia termed lethal reperfusion injury (Hearse & Bolli 1992, Yellon & Baxter 2000). There has been significant research over the last two decades focused on either pharmacological or mechanical ways to attenuate reperfusion injury and limit infarct size. Ischemic preconditioning phenomenon (whereby episodes of intermittent sublethal ischemia and reperfusion confer protection against a subsequent lethal episode of myocardial ischemia) which protects the myocardial tissue through adaptive mechanisms exerted primarily during ischemia (Murray *et al*., 1986) has been described twenty years ago. Although, preconditioning has been considered as a gold standard cardioprotective mechanism, its practical use in the clinical arena is limited by the inability to predict the onset of ischemia, which is the case in patients presented with myocardial infarction. However, the implementation of cardioprotective therapy at the time of reperfusion is clinically more feasible because the onset of reperfusion by either thrombolysis or PTCA is more

predictable and is under the clinician's control. Furthermore spontaneous reperfusion may occur in some patients before any hospital intervention due to dislodgment of the thrombus or relieved coronary spasm (Christian *et al.*, 1998). In this respect, the newly described phenomenon of ischemic postconditioning, in which the application of intermittent episodes of myocardial ischemia and reperfusion at the start of reperfusion period following a period of lethal ischemia confer a cardioprotective effect similar to ischemic preconditioning (Zhao *et al*., 2003), provides one such intervention. Reintroduction of mechanical ischemia at the time of reperfusion following a period of lethal ischemia may lead to potential complications, therefore, the concept of "pharmacological postconditioning", where pharmacological agents that mimic the effect of ischemic postconditioning is more clinically applicable.

Abundant research have shown that caspase mediated cell death (apoptosis) contributes significantly to cell death during ischemia reperfusion injury (Gottlieb *et al*., 1994, Saraste *et al*., 1997, Zhao *et al*., 2000) as has been discussed in chapter 1. Synthetic caspase inhibitors showed promising cardioprotective results. Broad spectrum caspase inhibitor (ZVAD) reduced infarction size and apoptotic cellular death when administered either during ischemia (Haung *et al*., 2000, Okamura et al., 2000, Holly et al., 1999) or shortly before starting reperfusion (Haung et al., 2000, Mocanu *et al*., 2000, McCully *et al*., 2004). However, specific caspase inhibitors showed contravesial results. Kovacs *et al* (2001) found that specific caspase 3 and 9 inhibitors failed to reduce infarction size or apoptotic cellular death after ischemia reperfusion injury when administed for only 10 minutes after starting reperfusion using langendorff working model. Okamura *et al* (2000) found that

administration of caspase-1 and caspase-3 inhibitors 5 minutes before ischemia resulted in significant reduction in apoptotic cell death, but they failed to show any significant change in infarct size using *in vivo* model. In contrast Mocanu *et al* (2000) found a significant reduction in infarct size after using caspase 8, 9 and 3 inhibitors, which were added at the time of reperfusion throughout using langendorff model. This discrepancy in results could be attributed to differences in timing of administration of the caspase inhibitors and models used.

Caspase inhibitors post reperfusion therapeutic window is not yet known. Previous literature showed controversial results, as Armstrong *et al* (2001) presented an abstract at the American Heart Association showing that caspase inhibitors (unspecified, still under investigation) significantly reduced infarct size even when administered one hour after the heart attack using *in vivo* rat model. However Li *et al* (2001) using isolated adult rabbit cardiomyocytes showed that caspase inhibitors (unspecified, labeled with numbers) were partially protective if added 15 min after reperfusion and the cardioprotective effect was completely lost with a delay of 30 or 60 minutes. This discrepancy could be attributed to the different inhibitors used (both used unknown inhibitors identified by numbers) and different models used. Therefore further research is needed to identify the effect of delayed administration of caspase inhibitors before applying them in the clinical field.

Therefore, the aim of this study is to determine whether broad spectrum caspase inhibitor (ZVAD) or specific caspase-3 inhibitor (DEVD) administered at different time points

during reperfusion (at start of reperfusion, 15 min, 30 min and 60 min after starting reperfusion) protect the rat myocardium from ischemia reperfusion injury using langendorff heart reperfusion model and isolated adult rat ventricular myocytes model.

The hypothesis of this study is that broad spectrum caspase inhibitor (ZVAD) or specific caspase inhibitor (DEVD) are cardioprotective at different time points during reperfusion through an anti-apoptotic pathway.

4.2 Methods

4.2.1 Langendorff heart reperfusion model (chapter 3 methods, section 3.4)

Isolated rat hearts were subjected to 20 min stabilisation, and then underwent 35 min regional ischemia followed by 120 min reperfusion using Langendorff perfusion system. Caspase inhibitors (Broad spectrum caspase inhibitor,ZVAD, 0.1 µM and Specific caspase-3 inhibitor, DEVD, 0.07 µM) were added at different time points during reperfusion (at start of reperfusion. 15 min, 30 min and 60 min after starting reperfusion). At the end of the experimental protocol, infarction size to risk area ratio (I/R %) determined using TTC staining. The haemodynamic parameters assessed were Left Ventricular Developed Pressure (LVDP, mmHg), Heart Rate (HR, beats per minute) and Coronary flow (CF, ml/min). These parameters were monitored throughout the experiment to monitor the hearts stability.

4.2.3 Analysis of isolated adult rat ventricular myocytes using flow cytometry (chapter 3 methods, section 3.6)

Isolated adult rat ventricular myocytes underwent 6 hours of hypoxia followed by 18 hours of reoxygenation. Caspase inhibitors (ZVAD, DEVD) were added at different time points during reoxygenation, at the start of reoxygenation, 15 min, 30 min and 60 min after starting reoxygenation following 6 hours of hypoxia. At the end of the experimental protocol, apoptotsis, necrosis, viability and intracellular caspase-3 activity were measured using flow cytometry analysis.

4.3 Results

4.3.1 Myocardial infarct size to risk area ratio (I/R %).

4.3.1.1 The effect of the broad spectrum caspase inhibitor (ZVAD; 0.1μM) on I/R % when added at different time points during reperfusion.

Previous research has shown that broad spectrum caspase inhibiton during either ischemia period or start of reperfusion period protects the myocardium from ischemia reperfusion injury. In the present study, we aimed at assessing whether delayed administration of the broad spectrum caspase inhibitor (ZVAD, 0.1 μM) at different time points during reperfusion period is still cardioprotective. Isolated perfused rat hearts underwent 35 minutes of ischemia followed by 120 minutes of reperfusion where ZVAD was administered at the start of reperfusion, 15 minutes, 30 minutes and 60 minutes after starting reperfusion throughout the reperfusion period. Infarct size is represented as the percentage of tetrazolium negative tissue in the ischemic risk zone. Results showed that ZVAD added at the start reperfusion significantly reduce I/R % by 37% when compared to control hearts (17.3 ± 2.0% *vs* 54.2 ± 6.6% control, Tukey's post-hoc test $p<0.001$) Figure 4.1. This significant cardioprotection was also observed when ZVAD was added at 15 min after starting reperfusion, I/R% was significantly reduced by 24% when compared to control hearts (30.5± 3.4% *vs* 54.2 ± 6.6 % control, $p<0.01$) Figure 4.1. Adding ZVAD 30 min and 60 min after starting reperfusion was still cardioprotective, I/R% significantly reduced by 23% when compared to control hearts (31.6 ± 4.0%, 31.5± 6.6 % respectively *vs* 54.2 ± 6.6 % control, Tukey's post-hoc test $p<0.01$) Figure 4.1. Statistical analysis also showed that, there was no significant difference between the different treatment groups when compared to each other (Tukey's post-hoc test $p>0.05$) Figure 4.1. This implies that

adding ZVAD at the start of reperfusion is equally cardioprotective as if added one hour after starting reperfusion.

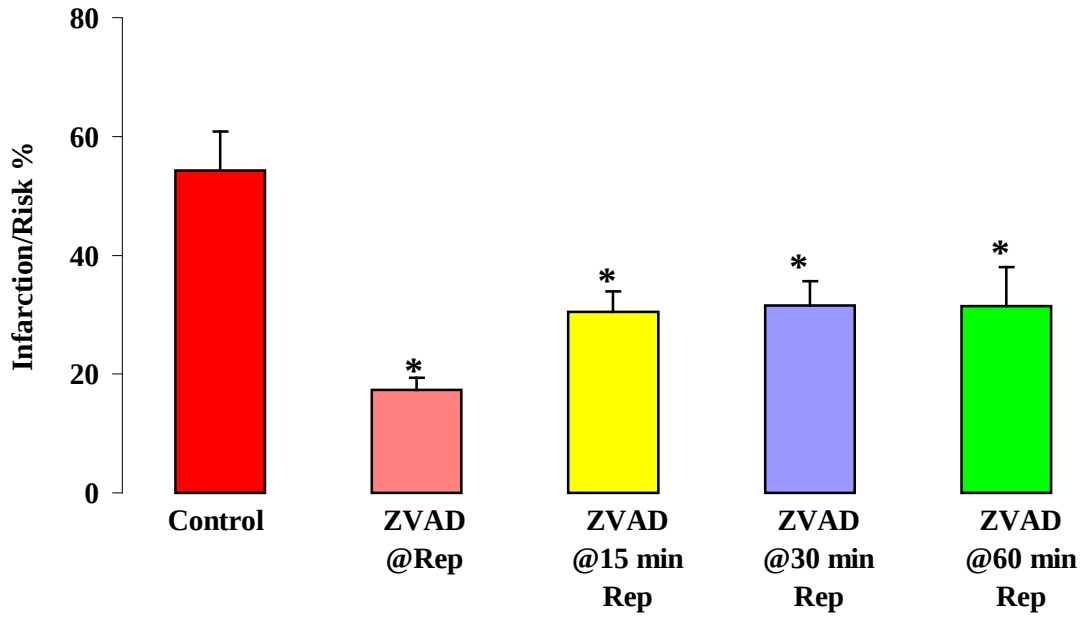


Figure 4.1: Infarct size to Risk ratio (%) in control and broad spectrum caspase inhibitor (ZVAD, 0.1µM) treated hearts. Isolated perfused rat hearts where subjected to 35 minutes of ischemia and 120 minutes of reperfusion where the ZVAD was administered at different time points during reperfusion. *P<0.01 *vs.* Control, Tukey's post-hoc test. Results are shown as Mean + SEM, n=5

4.3.1.2 The effect of the specific caspase-3 inhibitor (DEVD, 0.07µM) on I/R % when added at different time points during reperfusion.

Previous literature also showed promising cardioprotective results after using specific caspase inhibiton. In this study we aimed at assessing whether delayed administration of specific caspase-3 inhibitor (DEVD, 0.07 µM) is still cardioprotective. Isolated perfused rat hearts underwent 35 minutes of ischemia followed by 120 minutes of reperfusion where DEVD was administered at the start of reperfusion, 15 minutes, 30 minutes and 60 minutes after starting reperfusion throughout the reperfusion period. The percentage of tetrazolium-negative tissue in the ischemic risk zone is representative of the infarct size. The same

control ischemic group was used as ZVAD studies. The I/R% was significantly reduced by 40% in hearts treated with DEVD added at the start of reperfusion when compared to control hearts (14.4 ± 3.9 % *vs* 54.2 ± 6.6 % control; Tukey's post-hoc test $p<0.001$) Figure 4.2. Significant cardioprotection compared with control hearts was also found when DEVD was added at 15 min, 30 min and 60 min after starting reperfusion (15.7 ± 3.6%, 18.5 ± 3.7 and 26.5 ± 5.1 % respectively *vs* 54.2 ± 6.6 % control; Tukey's post-hoc test $p< 0.01$) Figure 4.2. There was no significant difference in I/R% when treatment groups were compared to each other (Tukey's post-hoc test $p>0.05$) Figure 4.2. The cardioprotection offered by broad spectrum caspase inhibitor (ZVAD) was not significantly different from the cardioprotection conferred by the specific caspase-3 inhibitor (DEVD) at all time points during reperfusion (Tukey's post-hoc test $p>0.05$).

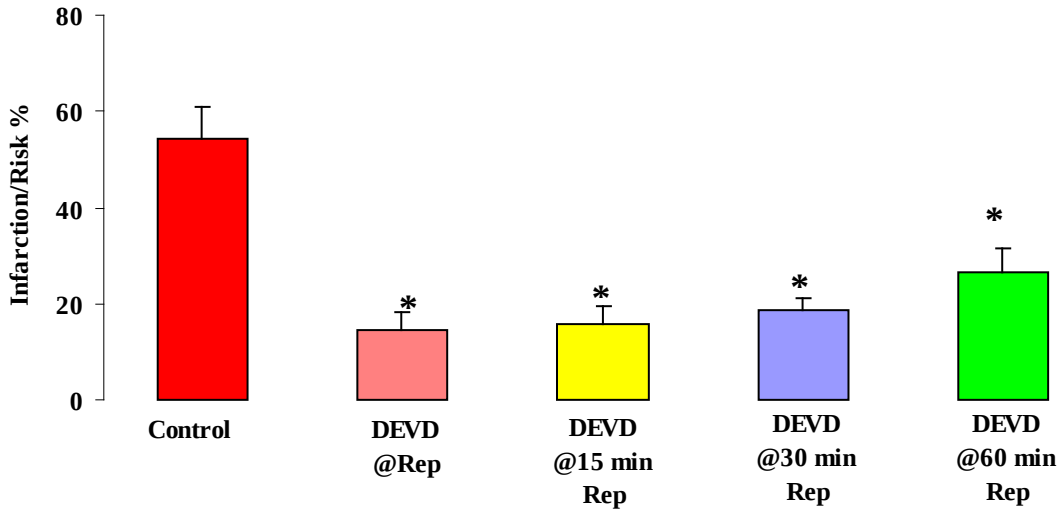


Figure 4.2: Infarct size to Risk ratio (%) in control and specific caspase-3 inhibitor (DEVD, 0.07µM) treated hearts. Isolated perfused rat hearts were subjected to 35 minutes of ischemia and 120 minutes of reperfusion. The DEVD-CMK (0.07µM) was administered at different time points during reperfusion. *P<0.01 vs. Control, Tukey's post hoc test. Results are shown as Mean + SEM, n=5

4.3.2 Haemodynamic parameters

4.3.2.1 Left ventricular developed pressure (LVDP)

LVDP was measured using inflated latex ballon inserted into the left ventricle and connected to a pressure transducer. LVDP was measured evry 5 minute throught the stabilisation, ischemia and reperfusion period. Results are expressed as % mean stabilisation LVDP and two- way ANOVA statistical analysis was used to assess for any significant difference between the experimental groups. % mean stabilization LVDP showed same general response outline in all the experimental groups. It dropped upon starting ischemia and showed significant decrease at the end of ischemia period when compared to stabilisation period. Upon starting reperfusion % mean stabilization LVDP increased, however it wasn't statistically significant when compared to end of ischemia period. Then it showed gradual drop tendency throughout reperfusion period. There was no significant difference in % mean stabilization LVDP between broad spectrum caspase inhibitor (ZVAD, 0.1 µM) treated groups when compared to control ischemia reperfusion group (ANOVA $P>0.05$) Figure 4.3. In specific caspase-3 inhibitor (DEVD, 0.07µM) treated experimental groups, there was no significant difference when compared to control ischemia group (ANOVA $p>0.05$) Figure 4.4.

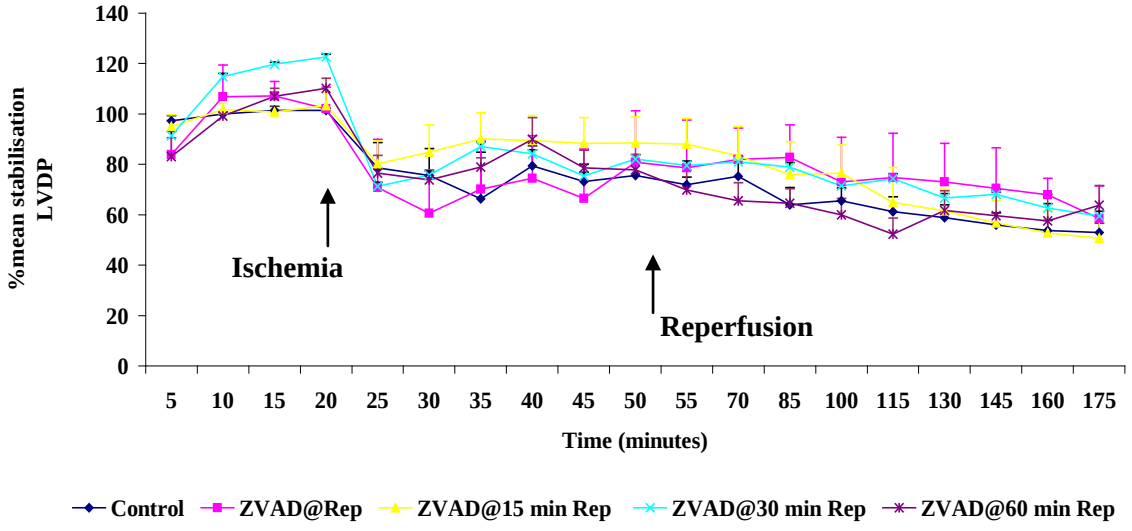


Figure 4.3: % mean stabilisation LVDP in control and broad spectrum caspase inhibitor (ZVAD, 0.1µM) treated hearts. Isolated perfused rat hearts subjected to 35 minutes of ischemia and 120 minutes of reperfusion where ZVAD was administered at different time points during reperfusion. Results are shown as mean percentages of the stabilisation period + SEM, n=5

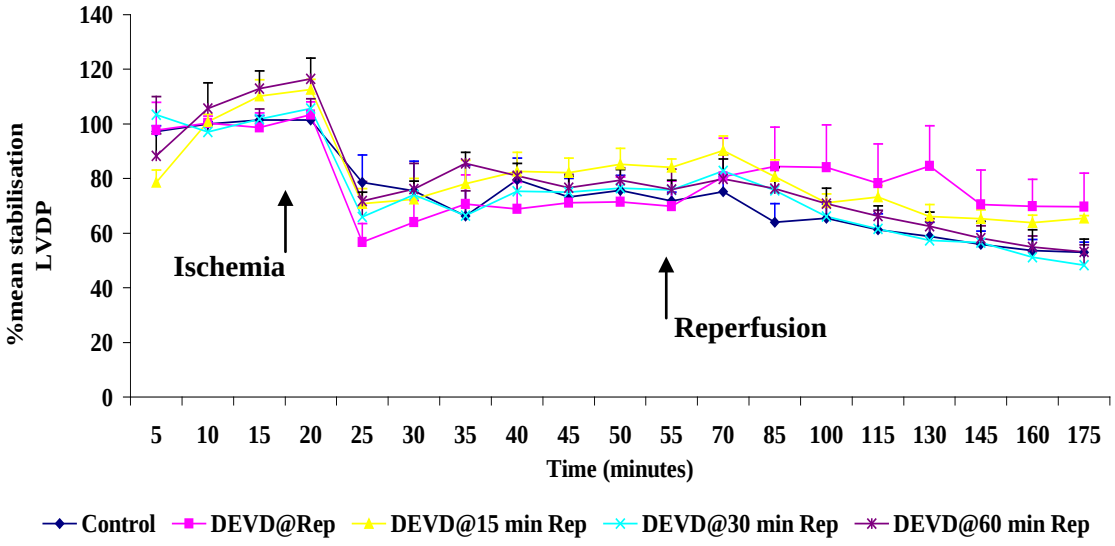


Figure 4.4: % mean stabilisation LVDP in control and the specific caspase-3 inhibitor (DEVD, 0.07µM) treated hearts. Isolated perfused rat hearts subjected to 35 minutes of ischemia and 120 minutes of reperfusion where DEVD was administered at different time points during reperfusion. Results are shown as mean percentages of the stabilisation period + SEM, n=5

4.3.2.2 Heart rate (HR)

HR was measured also through the inflated latex ballon inserted into the left ventricle and connected to a pressure transducer. HR showed a smaller response to ischemia and reperfusion when compared to LVDP and CF. It showed a stable response throught the experimental period. Results are expressed as % mean stabilisation HR. There was no significant differences in % mean stabilization HR between the different ZVAD treated experimental groups when compared to ischemia control group (ANOVA $p>0.05$) Figure 4.5. DEVD treated groups showed no significant difference when compared to control ischemia group (ANOVA $p>0.05$) Figure 4.6.

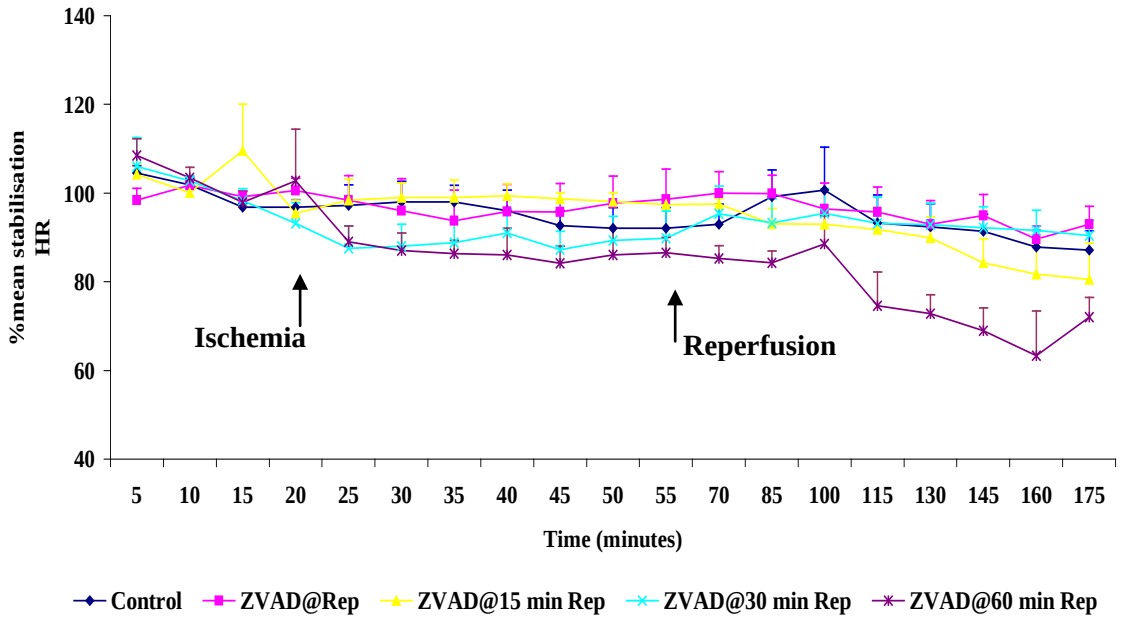


Figure 4.5: % mean stabilisation HR in control and broad spectrum caspase inhibitor (ZVAD, 0.1 µM) treated hearts. Isolated perfused rat hearts subjected to 35 minutes of ischemia and 120 minutes of reperfusion where ZVAD was administered at different time points during reperfusion. Results are shown as mean percentages of the stabilisation period + SEM, n=5.

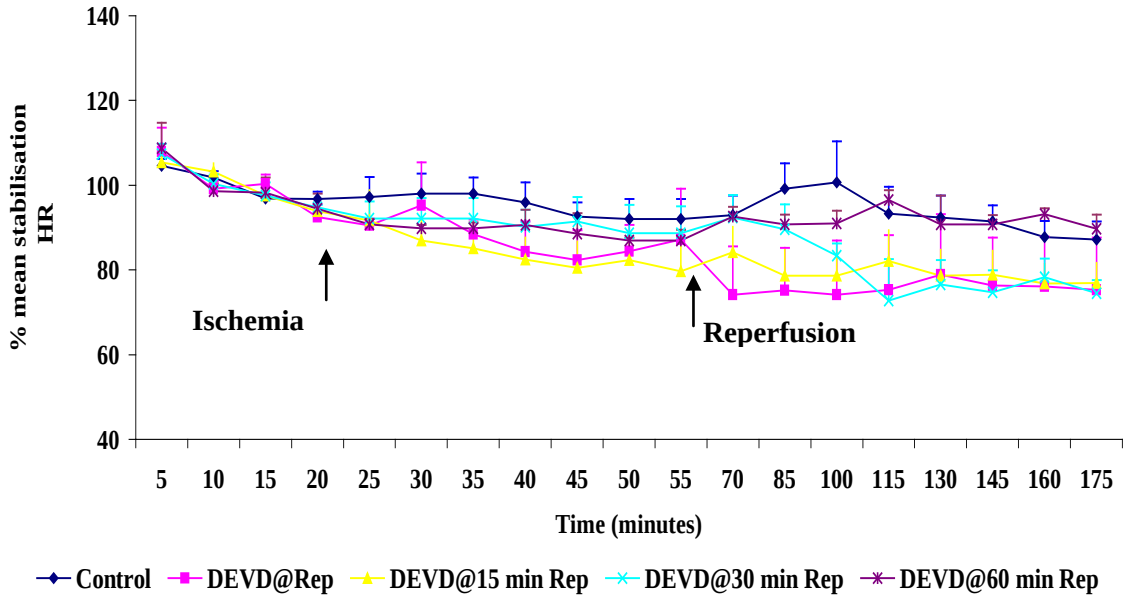


Figure 4.6: % mean stabilisation HR in control and the specific caspase-3 inhibitor (DEVD, 0.07µM) treated hearts. Isolated perfused rat hearts subjected to 35 minutes of ischemia and 120 minutes of reperfusion where the DEVD was administered at different time points during reperfusion. Results are shown as mean percentages of the stabilisation period + SEM, n=5.

4.3.2.3 Coronary flow (CF)

CF was stable during the stabilisation period and then decreased immediately during ischemia and it is a good indicator of ischemia. Upon starting reperfusion CF increased for the first 15 minutes, which thereafter showed a tendency to gradually decrease during the reperfusion period. This general response was observed in all the experimental groups. There were no significant differences in CF between the different experimental groups treated with ZVAD (0.1µM) or DEVD (0.07 µM) when compared to control (ANOVA $p>0.05$) Figure 4.7 and Figure 4.8.

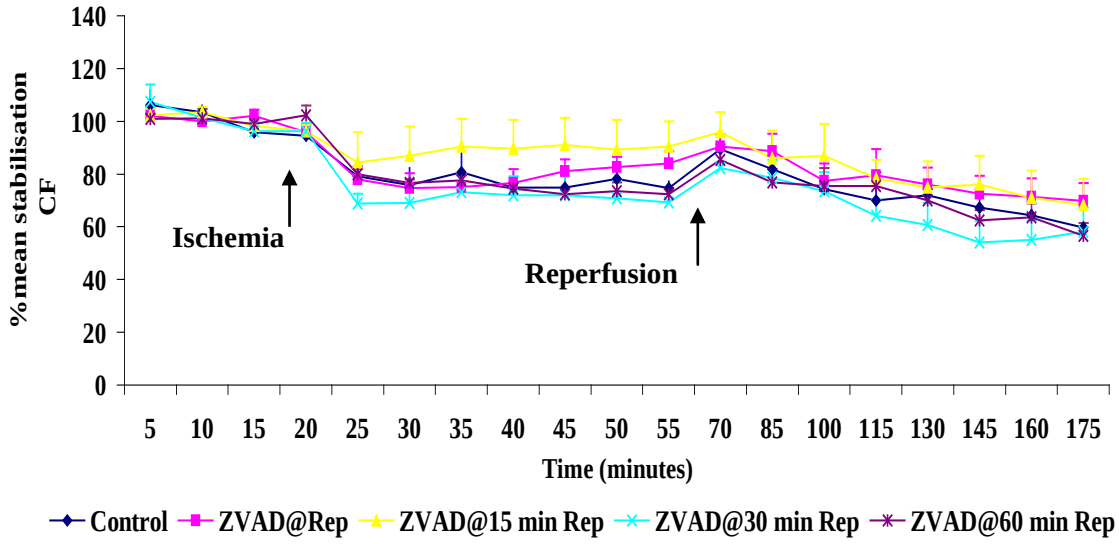


Figure 4.7: % mean stabilisation CF in control and broad spectrum caspase inhibitor (ZVAD, 0.1 µM) treated hearts. Isolated perfused rat hearts subjected to 35 minutes of ischemia and 120 minutes of reperfusion where the ZVAD was administered at different time points during reperfusion. Results are shown as mean percentages of the stabilisation period + SEM, n=5.

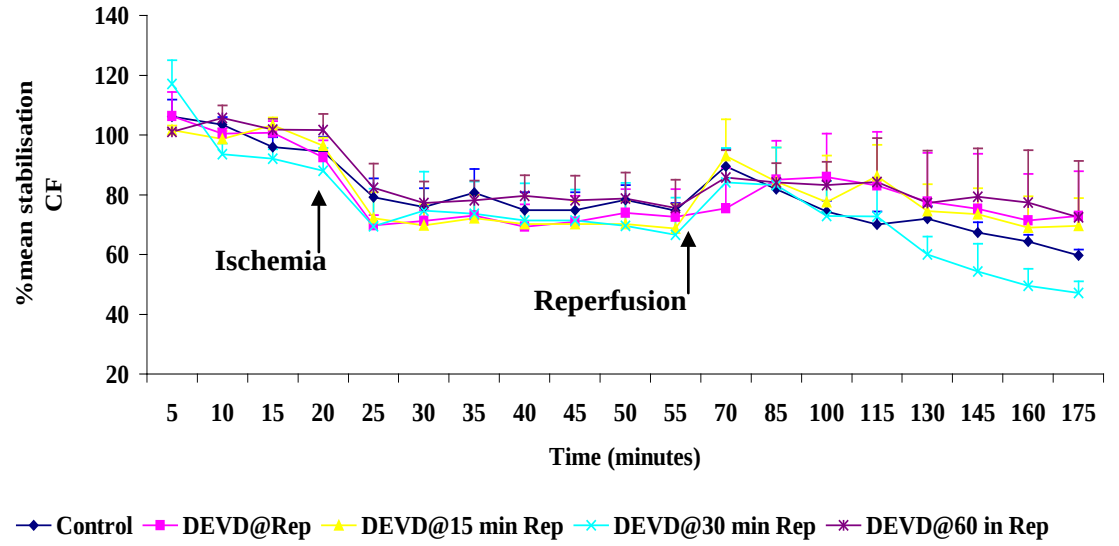


Figure 4.8: % mean stabilisation CF in control and the specific caspase-3 inhibitor (Ac-DEVD, 0.07µM) treated hearts. Isolated perfused rat hearts subjected to 35 minutes of ischemia and 120 minutes of reperfusion where the DEVD was administered at different time points during reperfusion. Results are shown as mean percentages of the stabilisation period + SEM, n=5.

4.3.3 Analysis of isolated adult rat ventricular myocytes using flow cytometry.

4.3.3.1 The effect of Hypoxia and Reoxygenation injury on viable, necrotic and apoptotic myocyte populations

Exposure of isolated adult rat ventricular myocytes to 6 hours of hypoxia followed by 18 hours of reoxygenation resulted in a significant reduction of cellular viabilty by 50% when compared to control normoxia cells, which had not been exposed to hypoxic conditions (18.2 ± 5.1 % *vs* 68.0 ± 2.9 % Normoxia, Tukey's post-hoc test $p<0.001$, Figure 4.8). Correspondingly there was a significant increase in necrotic cell death in the control group exposed to hypoxia and reoxygenation conditions when compared to the control normoxia cells (33.5 ± 2.2 % *vs* 18.2 ± 2.0 % Normoxia, Tukey's post-hoc test $p<0.01$, Figure 4.8). Hypoxia/Reoxygenation injury significantly increased the apoptotic cell population when compared to control normoxia cells not exposed to hypoxia and reoxygenation (48.2 ± 3.8 % *vs* 13.8 ± 3.0 % Normoxia, Tukey's post-hoc test $p<0.001$, Figure 4.8). The contribution of apoptosis to cellular death during hypoxia/reoxygenation injury was significantly more than necrosis by a factor of 15% (48.2 ± 3.8 % apoptosis *vs* 33.5 ± 2.2 necrosis, Tukey's post-hoc test $p<0.05$, Figure 4.8)

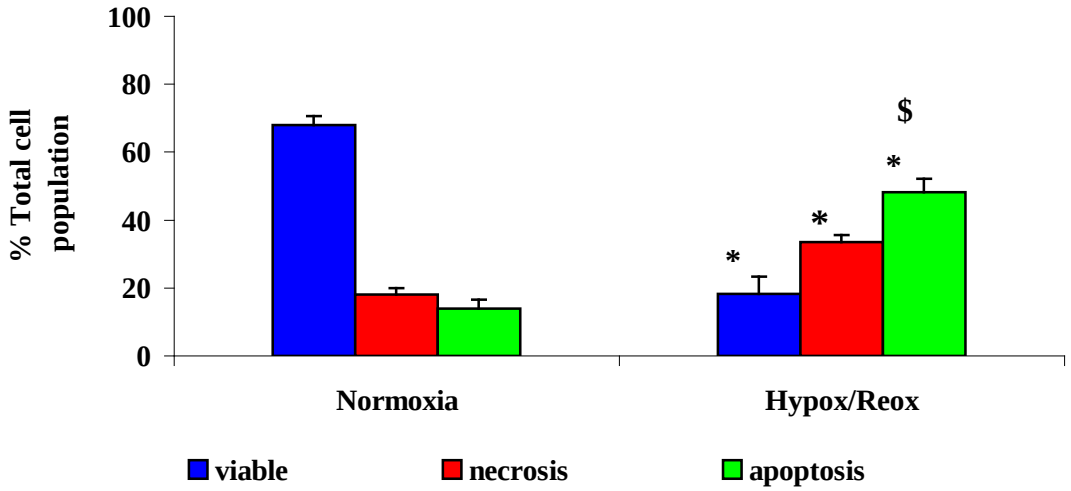


Figure 4.8: The effect of 6 hours hypoxia and 18 hours reoxygenation on adult rat ventricular myocyte viability, necrosis and apoptosis analysed by flow cytometry. * p < 0.05 *vs.* Normoxia, $ p<0.05 vs. apoptosis in Hypox/Reox group, Tukey's post-hoc test. Results are shown as means +SEM, n=5.

4.3.3.2 Preliminary results to decide the appropriate cardioprotective dose of the broad spectrum caspase inhibitor (ZVAD) and the specific caspase-3 inhibitor (DEVD) using adult rat ventricular myocyte model of hypoxia and reoxygenation

Preliminary studies using caspase inhibitors at low doses similar to langendorff model (ZVAD, 0.1 µM and DEVD, 0.07 µM) added at the start of reoxygenation showed no significant increase in cellular viability or significant decrease in necrosis and apoptosis cellular death when compared to control cells exposed to hypoxia and reoxygenation conditions ($p>0.05$, Figure 4.9, Figure 4.10). In previous studies investigating the cardioprotective effects of ZVAD and DEVD, high dose of 25 µM (Gottlieb *et al*., 1996, Kang *et al*., 2000, Hai *et al*., 2001) for both caspase inhibitors has been used. At this high dose, a significant increase in cellular viability was observed with both caspase inhibitors, similar to control normoxia group values. There was also a significant decrease in apoptotic and necrotic cellular death when compared to control hypoxia reoxygenation control group

(p< 0.05, Figure 4.9, Figure 4.10). Therefore 25µM dose of both ZVAD and DEVD was used throughout the isolated myocyte experiments.

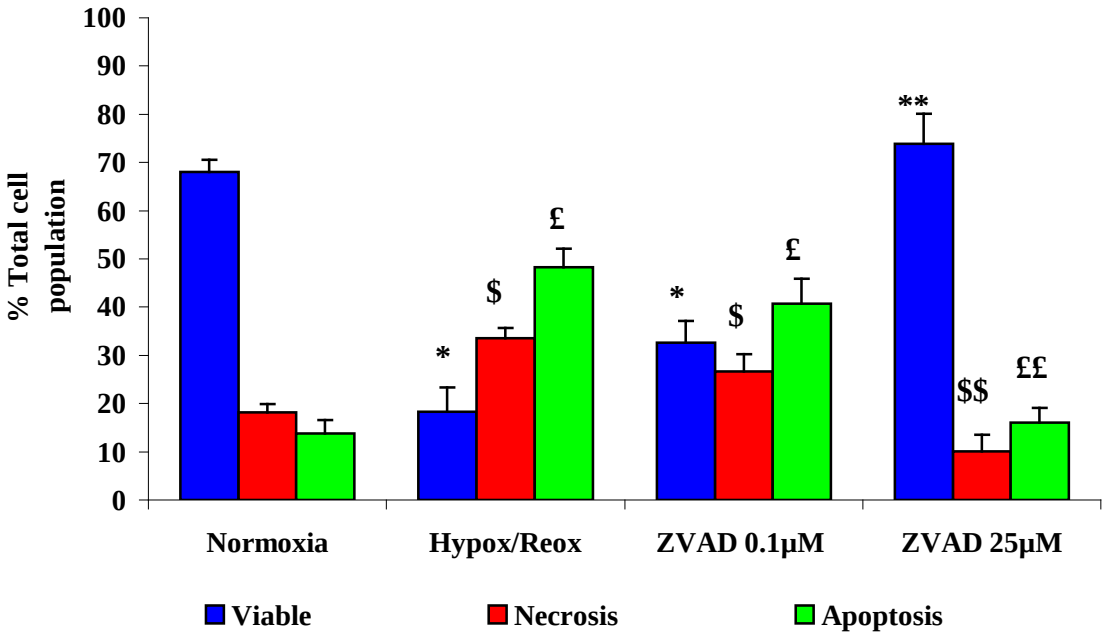


Figure 4.9: Assessment of cellular viability, necrosis and apoptosis in isolated adult rat cardiomyocytes subjected to 6 hours hypoxia and 18 hours of reoxygenation. The broad spectrum caspase inhibitor (ZVAD) was added at the onset of reoxygenation. Two doses of ZVAD were used (0.1µM, 25µM). Results are shown as mean + SEM and are expressed as a percentage of the total cells counted.* p<0.05 *vs*. Normoxia viable cells, $ p<0.05 *vs* Normoxia necrosis, £ p< 0.05 *vs*. Normoxia apoptosis, ** p< 0.05 *vs*. Hypox/Reox viable cells, $$ p< 0.05 Hypox/Reox necrosis, ££ p< 0.05 *vs*. Hypox/Reox apoptosis, n=4.

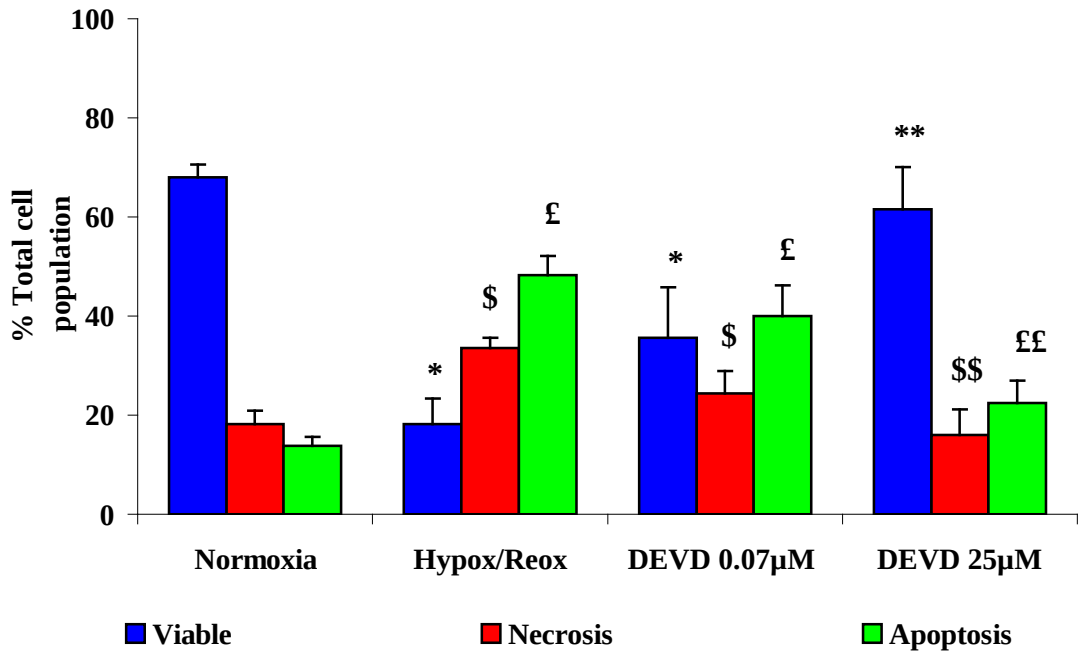


Figure 4.10: Assessment of cellular viability, necrosis and apoptosis in isolated adult rat cardiomyocytes subjected to 6 hours hypoxia and 18 hours of reoxygenation. The specific caspase-3 inhibitor (DEVD) was added at the onset of reoxygenation. Two doses of DEVD were used (0.07 µM, 25 µM). Results are shown as mean + SEM and are expressed as a percentage of the total cells counted.* p<0.05 *vs.* Normoxia viable cells, $ p<0.05 vs Normoxia necrosis, £ p< 0.05 *vs.* Normoxia apoptosis, ** p< 0.05 *vs.* Hypox/Reox viable cells, $$ p< 0.05 *vs.* Hypox/Reox necrosis, ££ p< 0.05 *vs.* Hypox/Reox apoptosis, n=4.

4.3.3.3 The effect of the broad spectrum caspase inhibitor (ZVAD, 25µM) on myocyte viability when added at different time points during reoxygenation

Same control Hypox.Reox group was used in all ARVMs studies. Adding ZVAD (25 µM) at the start of reoxygenation resulted in a significant increase in cellular viability almost to the same degree as normoxia control myocytes (73.9 ± 6.2 *vs* 68.0 ± 2.9 Normoxia, Tukey's post-hoc test p<0.05 Figure 4.11. Delayed administration of ZVAD (25 µM) 15 min, 30 min and 60 min after starting reoxygenation also resulted in significant increases in myocytes viability compared to Hypoxia/Reoxygentaion control myocytes (42.1 ± 6.5, 47.0 ± 3.6, 55.0 ± 4.3 respectively *vs* 18.2 ± 5.1 Hypox/Reox control, Tukey's post-hoc test

p<0.05) Figure 4.11. The cytoprotective effect of ZVAD was significantly greater when added at the start of reoxgenation than other delayed time points(15 min, 30 min and 60 min after starting reoxgenation) when compared to hypoxia/reoxygenation control myocytes (Tukey's post-hoc test p<0.05) Figure 4.11.

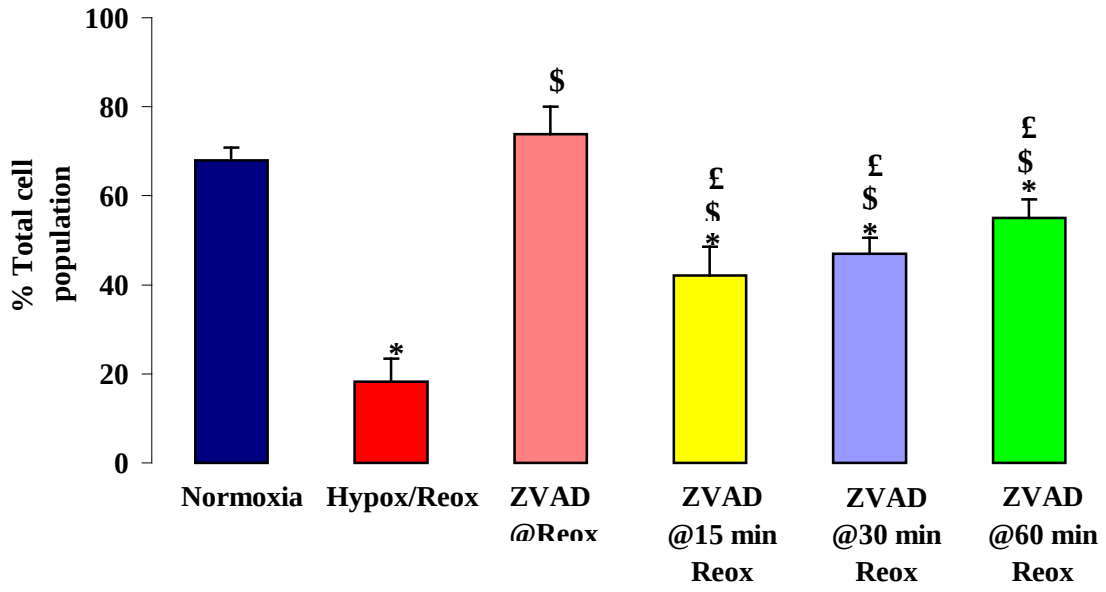


Figure 4.11: Assessment of myocytes viability in isolated adult rat cardiomyocytes subjected to 6 hours hypoxia and 18 hours of reoxygenation. The broad spectrum caspase inhibitor (ZVAD, 25µM) was added at different time points during reoxygenation. Results are shown as mean + SEM and are expressed as a percentage of the total cells counted.* p<0.05 *vs*. Normoxia, $ p<0.05 *vs* Hypox/Reox, £ p< 0.05 *vs*. ZVAD at Reox, n=5.

4.3.3.4 The effect of the broad spectrum caspase inhibitor (ZVAD, 25µM) on apoptotic myocyte death when added at different time points during reperfusion

ZVAD-fmk (25 µM) administered immediately at the start of reoxygenation and 15 min, 30 min and 60 minutes after starting reoxygenation resulted in a significant decrease in % apoptotic myocytes when compared to hypoxia/reoxygenation control myocytes (16.1 ± 3.0 %, 26.8 ± 1.7 %, 28.5 ± 1.9 %, 21.5 ± 2.3 % respectively *vs* 48.2 ± 3.8 % Hypox/Reox, Tukey's post-hoc test p<0.001) Figure 4.12. There was no significant difference in ZVAD

anti-apoptotic effect at different time points of administration (Tukey's post-hoc test p>0.05) Figure 4.12.

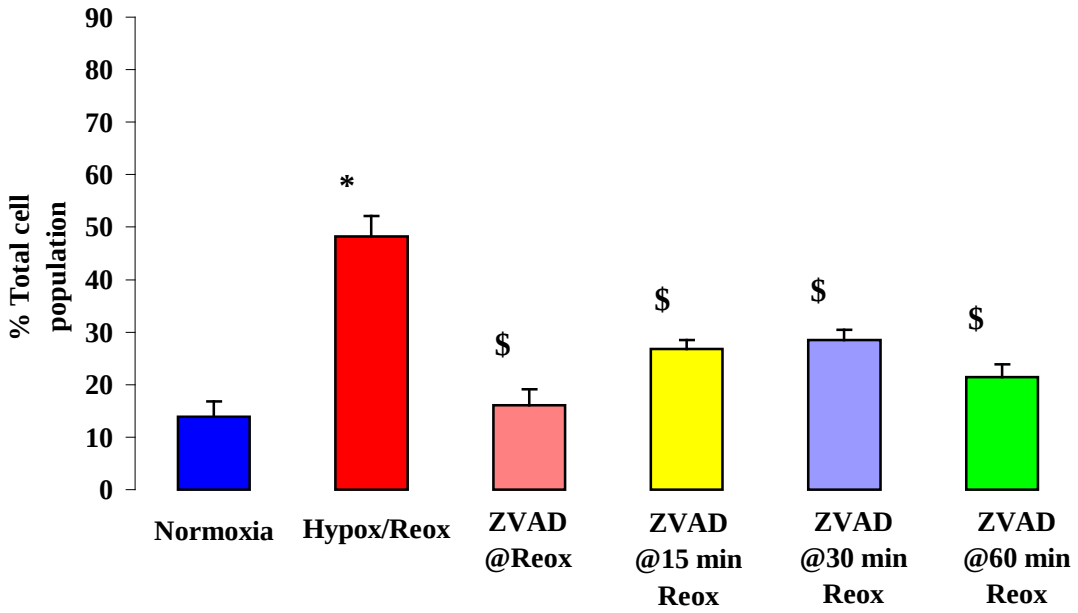


Figure 4.12: Assessment of myocytes apoptosis in isolated adult rat cardiomyocytes subjected to 6 hours hypoxia and 18 hours of reoxygenation. The broad spectrum caspase inhibitor (ZVAD, 25µM) was added at different time points during reoxygenation. Results are shown as mean + SEM and are expressed as a percentage of the total cells counted.* p<0.05 *vs*. Normoxia, $ p<0.05 *vs* Hypox/Reox, n=5.

4.3.3.5 The effect of the broad spectrum caspase inhibitor (ZVAD, 25µM) on necrotic myocyte death when added at different time points during reoxygenation

Adding ZVAD (25µM) at the start of reoxygenation period reduced the necrotic cell death significantly when compared to hypoxia/reoxygenation control myocytes (10.0 ± 3.4 *vs* 33.5 ± 2.2 %, Tukey's post-hoc test p<0.001) Figure 4.13. Delayed administration of ZVAD at 15 min, 30 min and 60 min after starting reoxygenation had no significant effect on necrotic cell death when compared to hypoxia/reoxygenation myocytes (31.0 ± 4.5, 24.4 ± 3.3, 23.5 ± 2.1 respectively *vs* 34.69± 2.27 % Hypox/Reox, Tukey's post-hoc test p>0.05) Figure 4.13.

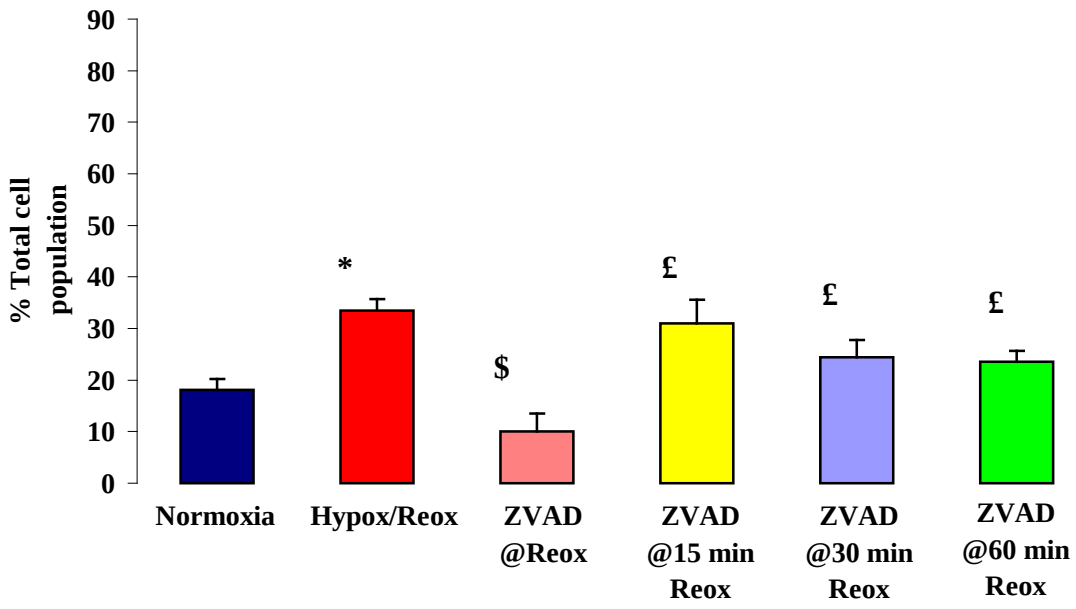


Figure 4.13: Assessment of myocyte necrosis in isolated adult rat cardiomyocytes subjected to 6 hours hypoxia and 18 hours of reoxygenation. The broad spectrum caspase inhibitor (ZVAD, 25µM) was added at different time points during reoxygenation. Results are shown as mean + SEM and are expressed as a percentage of the total cells counted.* p<0.05 *vs.* Normoxia, $ p<0.05 *vs* Hypox/Reox. £ p<0.05 *vs.* ZVAD at Reox, n=5.

4.3.3.6 The effect of the broad spectrum caspase inhibitor (ZVAD, 25µM) on intracellular Caspase-3 activity level when added at different time points during reperfusion

Caspase-3 is a pivotal effector caspase and an essential protease of the apoptotic machinery. Caspase-3 activity was measured using intracellular staining methods and analysed using flow cytometry. Isolated myocytes underwent 6 hours of hypoxia in 5% CO_2 0% O_2 followed by 18 hours of reoxygenation. ZVAD (25µM) was administered at the start of reoxygenation, 15 minutes, 30 minutes and 60 minutes after starting reoxygenation throughout. 6 hours hypoxia followed by 18 hours reoxygenation resulted in a significant increase in intracellular caspase-3 activity when compared to the control normoxia cells (375.47 ± 90.88 % *vs* 100 %, Tukey's post-hoc test p<0.001, Figure 4.14). ZVAD

significantly decreased caspase-3 activity when administered at all time points during reoxygenation (ZVAD at Reox 141.92 ± 26.63, ZVAD at 15 min Reox 123.84 ± 13.73%, ZVAD at 30 min Reox 138.50 ± 29.58 %, ZVAD at 60 min Reox 155.13 ± 21.35, vs Hypox/Reox 375.47 ± 90.88 , Tukey's post-hoc test $p<0.001$, Figure 4.14).

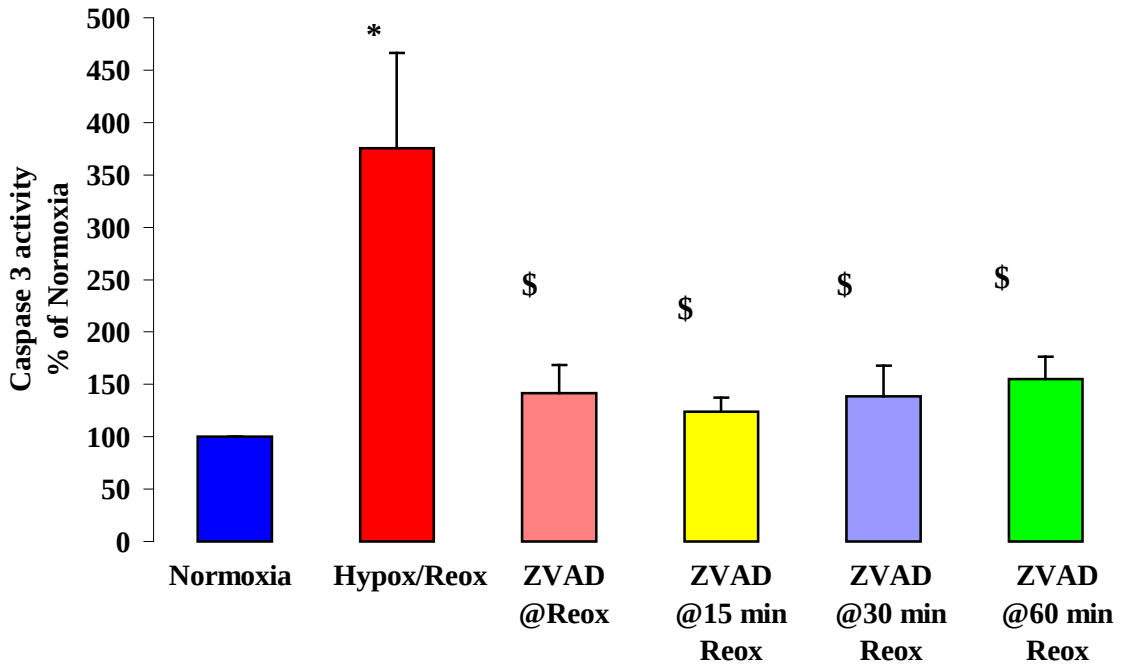


Figure 4.14. Caspase 3 activity in isolated adult rat cardiac myocytes subjected to 6 hours of hypoxia followed by 18 hours of reoxygenation. The broad spectrum caspase inhibitor (ZVAD, 25 µM) was administered at different time points during reoxygenation. *$p<0.05$ *vs.* Normoxia, $ $p<0.05$ *vs.* Hyp/Reox. Results are shown as means +SEM, n=6.

4.3.3.7 The effect of the specific caspase-3 inhibitor (DEVD, 25µM) on myocytes viability when added at different time points during reperfusion

DEVD (25µM) restored viable cells back to normoxia control values when added at the start of reoxygenation, 15 minutes, 30 minutes and 60 minutes after starting reoxygenation. This cytoprotection was significantly different to the hypoxia/reoxygention control myocytes (61.5 ± 8.6 % DEVD at Reox, 46.8 ± 3.3 % DEVD at 15 min Reox, 42.8 ± 6.5

DEVD at 30 min Reox, 53.4 ± 3.8 DEVD at 60 min Reox *vs* 18.2 ± 2.6 % Hypox/Reox, Tukey's post-hoc test p<0.05) Figure 4.15.

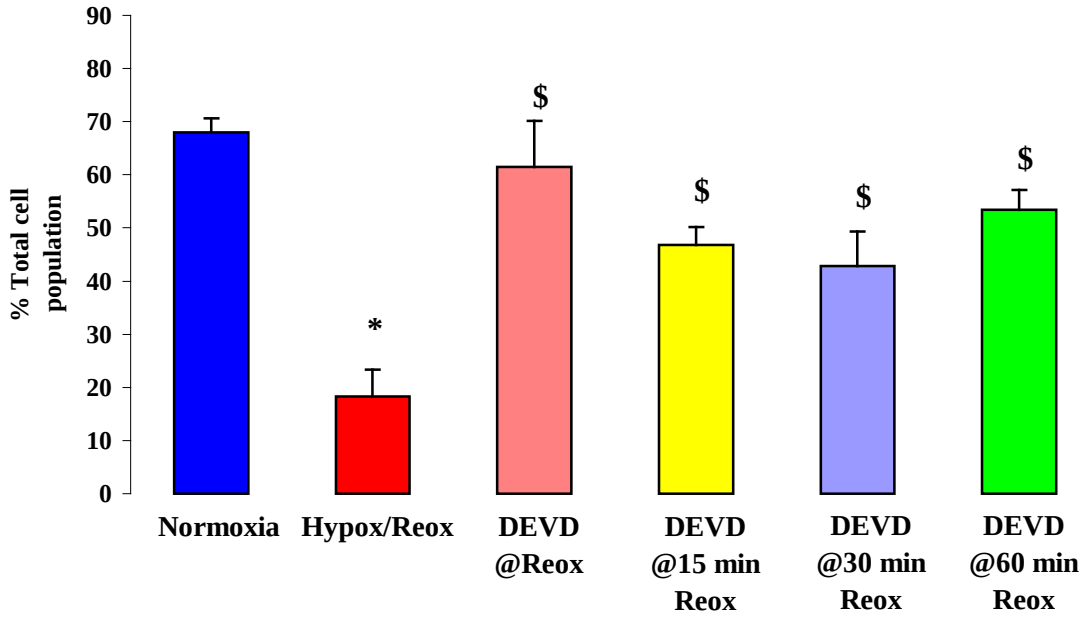


Figure 4.15: Assessment of cellular viability in isolated adult rat cardiomyocytes subjected to 6 hours hypoxia and 18 hours of reoxygenation. The specific caspase-3 inhibitor (DEVD, 25µM) was added at different time points during reoxygenation. Results are shown as mean + SEM and are expressed as a percentage of the total cells counted.* p<0.05 *vs*. Normoxia, $ p<0.05 *vs* Hypox/Reox, n=5.

4.3.3.8 The effect of the specific caspase-3 inhibitor (DEVD, 25µM) on apoptotic myocyte death when added at different time points during reoxygenation

DEVD (25µM) significantly reduced the apoptotic cell population to almost the normoxia control cells level when administered at the start of reoxygenation, 15 minutes, 30 minutes and 60 minutes after starting reoxygenation (22.4 ± 4.6 % DEVD at Reox, 18.9 ± 5.2 % DEVD at 15 min Reox, 28.8 ± 5.5 % DEVD at 30 min Reox, 23.3 ± 4.5 % DEVD at 60 min Reox *vs* 48.2 ± 3.8 % Hypox/Reox, Tukey's post-hoc test p<0.05) Figure 4.16.

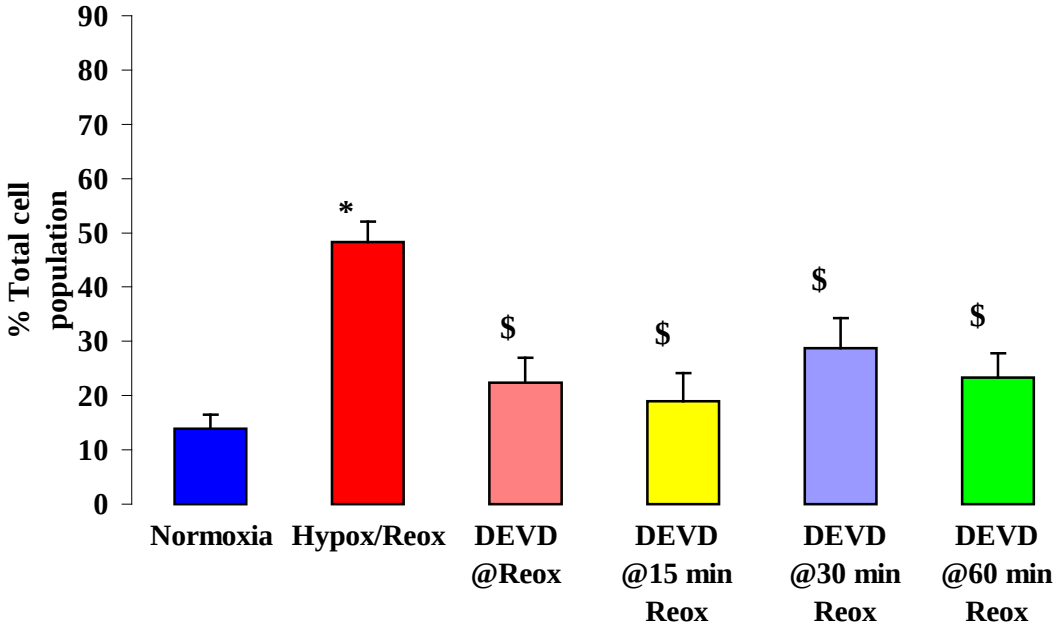


Figure 4.16: Assessment of cellular apoptosis in isolated adult rat cardiomyocytes subjected to 6 hours hypoxia and 18 hours of reoxygenation. The specific caspase-3 inhibitor (DEVD, 25µM) was added at different time points during reoxygenation. Results are shown as mean + SEM and are expressed as a percentage of the total cells counted.* p<0.05 *vs*. Normoxia, $ p<0.05 *vs* Hypox/Reox, n=5.

4.3.3.9 The effect of the specific caspase-3 inhibitor (DEVD, 25µM) on necrotic myocytes death when added at different time points during reperfusion

Adding DEVD (25µM) at the start of reoxygenation period significantly reduced the % necrotic cells when compared to the Hypox/Reox control myocytes (16.0 ± 5.2 *vs* 33.5 ± 2.2 %, Tukey's post-hoc test P< 0.05) Figure 4.17. There was no significant effect of delayed administration of DEVD at 15, 30 or 60 minute after starting reoxgenation on % necrotic myocytes when compared to the Hypox/Reox control myocytes (34.3 ± 4.5%, 28.4 ± 6.5%, 22.3 ± 3.8 % respectively *vs* 33.5 ± 2.2 % Hypox/Reox, Tukey's post-hoc test p> 0.05) Figure 4.17.

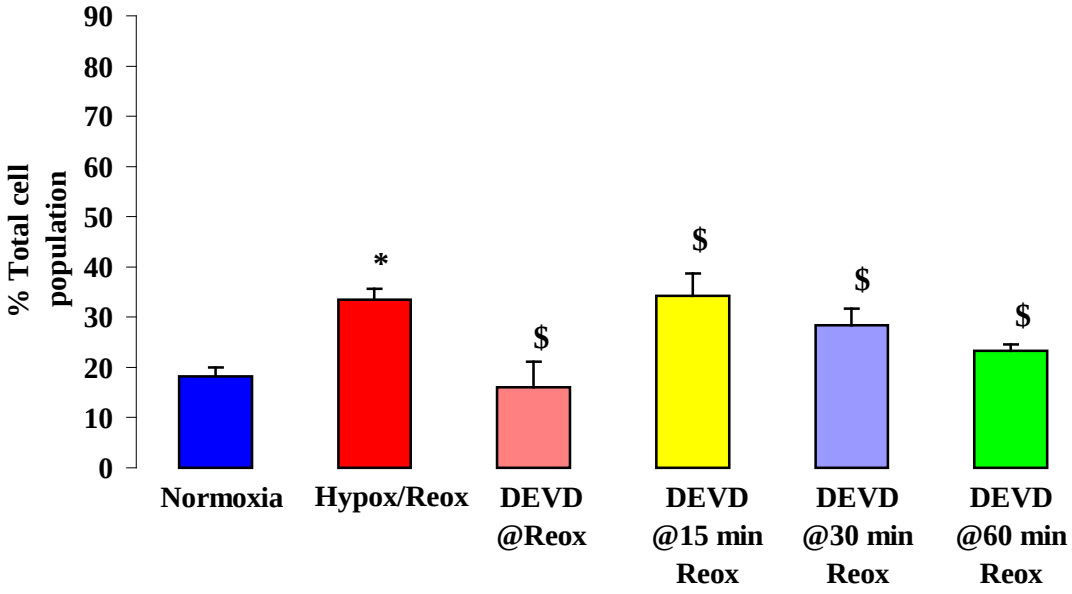


Figure 4.17: Assessment of cellular necrosis in isolated adult rat cardiomyocytes subjected to 6 hours hypoxia and 18 hours of reoxygenation. The specific caspase-3 inhibitor (DEVD, 25µM) was added at different time points during reoxygenation. Results are shown as mean + SEM and are expressed as a percentage of the total cells counted.* p<0.05 *vs*. Normoxia, $ p<0.05 *vs.* Hypox/Reox, n=5.

4.3.3.10 The effect of the specific caspase-3 inhibitor (DEVD, 25µM) on intracellular Caspase-3 activity level when added at different time points during reperfusion

Hypoxia/Reoxygenation injury resulted in a significant increase in caspase-3 activity when compared to the control normoxia cells (375.47 ± 90.88 % vs 100 %, Tukey's post-hoc test p<0.001) Figure 4.18. DEVD (25µM) significantly decreased caspase-3 activity when administered at the start of reoxygenation, 15 minutes, 30 minutes and 60 minutes after starting reoxygenation (DEVD at Reox 127.52 ± 21.19, DVD at 15 min Reox 145.18 ± 54.38 %, DEVD at 30 min Reox 136.03 ± 22.86 %, DEVD at 60 min Reox 155.20 ± 33.37 %, *vs* Hypox/Reox 375.47 ± 90.88, Tukey's post-hoc test p<0.01) Figure 4.18.

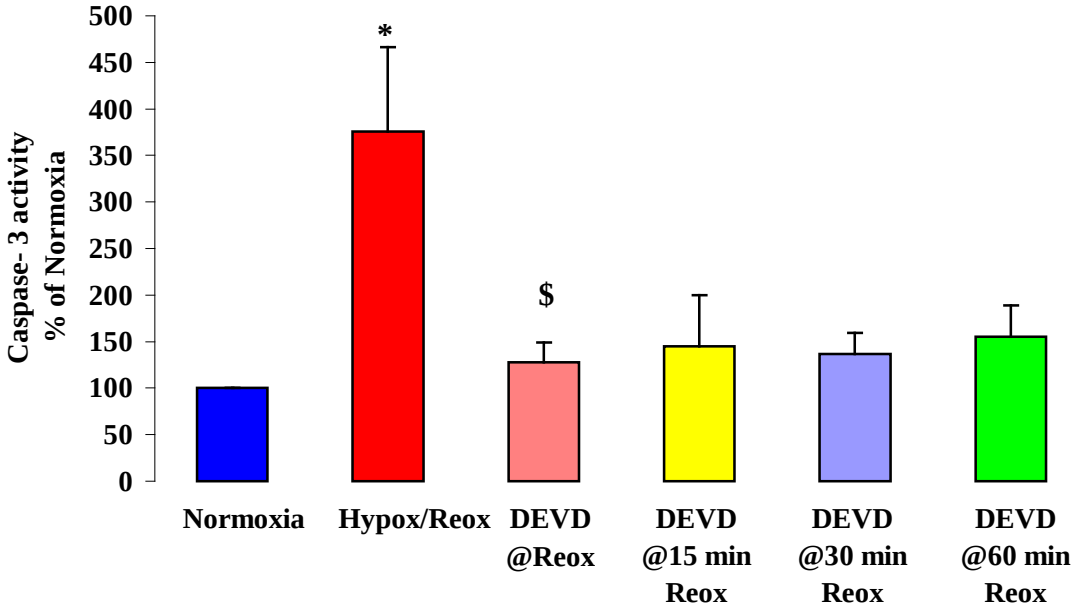


Figure 4.18: Caspase 3 activity in isolated adult rat cardiac myocytes subjected to 6 hours of hypoxia followed by 18 hours of reoxygenation. The specific caspase 3 inhibitor (DEVD, 25 µM) was administered at different time points during reoxygenation. *p<0.05 *vs.* Normoxia. $ p<0.05 *vs.* Hyp/Reox. Results are shown as means +SEM, n=6.

4.3.3.11 Results summary

In summary both the broad spectrum caspase inhibitor (ZVAD) and the specific caspase 3 inhibitor (DEVD) resulted in significant cardioprotection by siginicant reduction in infarction size within the risk area, significant reduction in apoptotic and necrotic cellular death and siginificant reduction in intracellular caspase 3 acitivity. This cardioprotection was observed at all times of administration as shown n table 4.1. Same control ischemic group was used for all langendorff experiments and same control hypox/reox group was used in all ARVMs experiments

Group	I/R %	Viability	Apoptosis	Necrosis	Caspase 3 activity (%of Normoxia)
Normoxia		68.0± 2.9	13.8± 3.0	18.2 ±2.0	100%
Control Ischemia/Rep	54.2±6.6	18.2±5.1	48.2± .8	33.5± 2.2	375.5±90.88
ZVAD@Rep	17.3±2.0*	73.9±6.2*	6.1±3.0*	10.0±3.4*	141.9±26.6*
ZVAD@15min Rep	30.5±3.4*	42.1± 6.5*	26.8±1.7*	31.0±4.5	123.8±13.7*
ZVAD@30min Rep	31.6±4.0*	47.0±3.6*	28.5±1.9*	24.4±3.3	38.5±29.6*
ZVAD@60min Rep	31.5±6.6*	55.0±4.3*	21.5±2.3*	23.5±2.1	155.1± 1.4*
DEVD@Rep	14.4±3.9*	61.5±8.6*	22.4±4.6*	16.0±5.2*	127.5±21.2*
DEVD@15min Rep	15.7±3.6*	46.8±3.3*	18.9±5.2*	34.3±4.5	145.2±54.4*
DEVD@30min Rep	18.5±3.7*	42.8±6.5*	28.8±5.5*	28.4±6.5	136.0±22.9*
DEVD@60min Rep	26.5± 5.1*	53.4 ±3.8*	23.3± 4.5*	22.3 ±3.8	155.2±33.4*

Table 4.1 Summary table showing the effect of the broad spectrum caspase inhibitor (ZVAD) and the specific caspase 3 inhibitor (DEVD) on I/R%, cellular viability, apoptosis, necrosis and intracellular caspase 3 activity. Results are shown as mean ± SEM * p<0.05 vs. control ischemia reperfusion.

4.4 Discussion

4.4.1 Cardioprotective effect of caspase inhibitors when administered at different time points during reperfusion

This study demonstrated that, the broad spectrum caspase inhibitor (ZVAD) and the specific caspase-3 inhibitor (DEVD) were cardioprotective when introduced immediately at the start of reperfusion by reducing infarct size through an anti-apoptotic and anti-necrotic effect. Delayed administration of aforementioned caspase inhibitors at 15 min, 30 min and 60 min after starting reperfusion were still cardioprotective by reducing infarct size through an anti-apoptotic effect.

This cardioprotective effect of the aforementioned caspase inhibitors when introduced immediately after starting reperfusion were in agreement with previous studies (Mocanu *et al.*, 2000, McCully *et al*., 2004), where they showed cardioprotective effect of caspase inhibitors when introduced immediately after starting reperfusion. Studies investigating the effect of introducing caspase inhibitors after starting reperfusion are limited in number and their results are contradictory. Armstrong *et al.,* (2001) presented an abstract at the American Heart Association showing that unidentified caspase inhibitor (IDN6734) reduced infarct size up to 50% when added at the start of reperfusion and only by 27% when administered one hour after the heart attack using rat *in vivo* model. However Li *et al.,* (2001) using isolated adult rabbit cardiomyocytes showed that caspase inhibitors (unidentified) were partially protective if added 15 min after reperfusion but that effect was completely lost with a delay of 30 or 60 minutes after the start of reperfusion. In our study we showed that both broad spectrum and specific caspase-3 inhibitors were

cardioprotective even when administered one hour after reperfusion. Furthermore, this study compared the effect of these caspase inhibitors at different time points during reperfusion, to show that caspase inhibitors introduced one hour after reperfusion are almost as cardioprotective as if they were introduced at the time reperfusion started.

We observed a dose dependent effect of ZVAD and DEVD on its ability to increase cellular viability and decrease cellular necrosis and apoptosis using adult rat ventricular myocytes model (ARVMs) when compared to their cardioprotective effect using langendorff model. A high dose of 25µM was required to show significant cardioprotection using ARVMs model which is the lowest effective dose found by a previous study using similar model (Kang *et al*., 2000). The effective inhibitory concentration of caspase inhibitors is variable among different studies, cytoprotective effect was showed at a doses ranging from 10µM - 100µM using the ARVMs model of hypoxia/Reoxygenation (Gottlieb *et al*., 1996, Kang *et al*., 2000, Hai *et al*., 2001), on the other hand infarction size was significantly reduced at lower doses of the caspase inhibitors as 0.07µM and 0.1µM (Mocanu *et al*., 2000). This variability could be attributed to the different models used and to the prolonged reperfusion period in ARVMs model when compared to langendorff model. Isolated hearts perfused using Langendorff model is viewed as an invaluable and first choice *in vitro* model in the development and assessment of anti-ischemic protective interventions (Hearse & Suttherland, 2000), however the maintainance of cellular homeostasis is limted and porn to deterioration over short time (Ytrhus, 2000). ARVMs model allows studying ischemia reperfusion injury over extended time course when we compare it to langendorff model (Ytrhus, 2000). Furthermore it allows measurment of differential cellular death types.

Therefore using these two models in our study is essential to expand our knowledge about the role of caspase inhibitors in myocardial ischemia reperfusion injury befor applying them in clinical field.

4.4.2 Differential contribution of apoptosis and necrosis to ischemia reperfusion injury and the effect of caspase inhibitors when administered at different time points during reoxygenation

The results of this study showed that ischemia reperfusion of the cardiomyocytes resulted in a significant increase in apoptosis, necrosis and caspase-3 activity level. The individual contribution of apoptosis and necrosis to cell death during myocardial ischemia reperfusion injury and at what point they contribute to myocyte death is still controversial (Gottlib & Engler, 1999). In our model of hypoxia/reoxygenation using adult rat ventricular myocytes, results showed a significant increase in both apoptotic and necrotic myocyte death with hypoxia/reoxygenation injury when compared to myocytes under normal conditions. This implies that both types of myocyte death are involved in ischemia reperfusion injury, these results were in agreement with previous studies (Buja & Entman, 1998, Yoaita *et al*., 1998, Fliss *et al*., 1996). Interestingly, this study showed that apoptosis contribution to myocytes death during hypoxia reoxygenation injury is significantly greater than necrotic myocyte death. However McCully *et al*., (2004) showed that contribution of necrosis to infarct size is greater than apoptosis using rabbit isolated hearts. They might be underestimated apoptosis in their 3 hours reperfusion model compared to 18 hours of oxygenation in our model, as previous studies have shown that apoptosis increased progressively with time after starting reperfusion and peaked out within several hours after myocardial ischemia (Anversa *et al*., 1998, Buja & Entman 1998).

Both Caspase inhibitors when administered at the start of reperfusion in this study resulted in a significant reduction of infarct size which could be attributed to a significant increase in cell viability, significant decrease in apoptosis and significant decrease in necrosis. However infarction reducing effect of delayed administration of caspase inhibitors 15 min, 30 min and 60 min after starting reperfusion was mainly through anti-apoptotic effect without any significant change in necrosis. The anti-apoptotic and anti-necrotic effect of caspase inhibitors in this study was in agreement with previous studies, where it has been suggested that caspase inhibitors might have both anti apoptotic and anti-necrotic effect; furthermore it was revealed that caspase inhibitors retarded necrotic cell death as well as apoptotic cell death in an *in vitro* system of chemical hypoxia (Shimizu *et al*., 1996 a, Shimizu *et al*., 996b, Shimizu *et al*., 1996c). This significant cardioprotection by caspase inhibitors through anti-apoptotic effect without any anti-necrotic effect when administered at delayed time points during reperfusion highlighted the significant role played by apoptosis in exacerbation and extension of ischemia reperfusion injury. This also indicates that apoptosis contribution to cellular death during ischemia reperfusion injury is significantly more at later time points after starting reperfusion. In addition this provides evidence that caspase activation, which might be initiated during ischemia, but their irreversible cellular injury, does not occur until later time during reperfusion. This is supported by a study by Vanden Hoek *et al*., (2003), where they reported that at least 2 hours of reperfusion was required to detect a significant level of caspase-3 activity required for fodrin cleavage to be detected.

The similar significant cardioprotection through anti-apoptotic effect offered by the broad spectrum and selective caspase inhibitors in this study raises the possibility that these compounds might have non-specific actions. Berger *et al*., (2006) tested different selective caspase inhibitors and the results showed that none of the compounds are highly selective and all exhibit broad inhibition and cross reactivity. In addition even though both caspase inhibitors in our study resulted in a significant decrease in caspase-3 activity (downstream effector caspase), they might have an inhibitory effect on other proteases implicated in myocardial ischemia reperfusion injury such as calpains (Yoshida *et al*., 1995, 1999, Iwamoto *et al*., 1999, Khalil *et al*., 2005). Calpains, which are also cysteine proteases and share many common substrates with caspase 3 (Wang, 2000), are involved in both necrotic and apoptotic cell death as shown by McGinnis *et al*., (1999). Calpain inhibitors have also been shown to reduce ischemia reperfusion injury (Perrin *et al*., 2003, Khalil *et al*., 2005). Therefore the inhibitory effect of caspase inhibitors on Calpains can not be ruled out in this study. In addition caspase inhibitors have also been found to efficiently inhibit cathepsin B activity in vitro and in tissue culture cells (Schotte *et al*., 1998, Gray *et al*., 2001), which is another cystein protease enzyme that has already been, implicated in some apoptotic models (Guicciardi *et al*., 2000, Stoka *et al*., 2001). Therefore non-caspase related mechanisms should be considered when interpreting the effect of caspase inhibitors.

4.4.3 The effect of caspase inhibitors on heart haemodynamics when added at different time points during reperfusion

An analysis of hemodynamic parameters were taken in the isolated perfused rat heart langendorff studies in order to determine if there were any functional changes occurring with caspase inhibitors treatment. In this study we found that both broad spectrum caspase inhibitor (ZVAD) and specific caspase-3 inhibitor (DEVD) had no significant effect on systemic hemodynamic, LVDP and HR or on coronary flow compared to control ischemia/reperfusion hearts. Insignificant effect of caspase inhibitors on functional recovery has been shown by different studies using langendorf model (Mocanu *et al*., 2000, McCully *et al*., 2004). Using langendorff perfusion model; functional parameters declined steadily during the experimental time course even without any ischemic intervention (Mocanu *et al*., 2000), therefore it is not a truly reliable model to assess functional recovery of the hearts post treatment.

4.4.4 Clinical implications of the study

Clinically, patients rarely present to hospital before the onset of ischemia (heart attack), however they usually seek medical attention after the onset of ischemia and reperfusion. Therefore, the implementation of cardioprotective therapy at the time of reperfusion is clinically feasible because the onset of reperfusion is more predictable and is under the clinician's control. In comparison to well established and gold standard ischemic preconditioning which protects myocardial tissue through adaptive mechanisms exerted primarily during ischemia (Murray *et al*., 1986), postconditioning applied at the onset of reperfusion, exerts protection during very early reperfusion (Zhao *et al*., 2003). Ischemic

postconditioning is a new evolving and promising area in clinical medicine, introducing drugs after starting reperfusion as indicated in our study can be considered as a form of pharmacological postconditioning which is more clinically feasible for patients. The results of our study, suggest that delayed administration of caspase inhibitors after reperfusion (as in the clinical setting of heart attack), may still salvage myocardium. In addition, the results of our study provide evidence that the therapeutic window of myocardial infarction treatment can be prolonged for at least an hour after starting reperfusion using caspase inhibitors, which is again more clinically applicable. This implies that the therapeutic window for caspase inhibition in myocardial ischemia reperfusion treatment is longer than for most cardioprotective drugs tested. Hussain (2006) showed that administration of A_3 agonist CL-IB-MECA 15 min and 30 min after starting reperfusion is still cardioprotective, howeverer cardioprotection was lost with delayed administration of 60 min after starting reperfusion. Boucher *et al*., (2004) reported that delaying administration of A_{2a} agonist 5 minutes after reperfusion abolishes its protective effect seen when administered at the start of reperfusion. Jonassen *et al*., 2000 showed that administration of hormone insulin 15 minutes after the onset of reperfusion resulted in a loss of the protection seen when administered at reperfusion.

The effective inhibitory dose of caspase inhibitors varied betweeen models used as we mentioned above. Using high dose of caspase inhibitors might have unwanted side effects when applied in clinical field. As apotosis and caspase activation occurred in pathological as well as physiological conditions, therefore applying caspase inhibitors in high doses might affect more essential physiological process leading to more unwanted side effect.

Therefore further studies needed to evaluate the effective inhibitory dose to be used in ischemic heart disease treatment with inimal unwanted side effect.

4.4.5 Conclusion

In conclusion, this study showed that caspase inhibitors significantly reduced myocardial infarction size and increased cellular viability through an anti-apoptotic effect via inhibition of downstream effector caspase-3, when administered at different time points during reperfusion. This implies that the therapeutic window of caspase inhibitors could be prolonged for at least one hour after starting reperfusion, which is more clinically applicable time frame in treating patients with myocardial infarction. Furthermore, this study adds to current literature further evidencing about the significant role played by apoptosis in myocardial ischemia reperfusion injury.

CHAPTER 5

Myocardial Protection during Early Reperfusion by Administration of Caspase Inhihibitors is mediated via PI3-Akt Survival Pathway

5.1 Introduction and objectives

The caspase are group of cysteine proteases, which represent the executionary machine of apoptosis (Cryns & Yaun, 1998). Caspase, can be activated by two main pathways, the first is mediated by cell surface death receptors, leading to autoactivation of caspase-8, which in turn activates downstream effector caspase such as caspase-3, 6 and 7 (Budihardjo *et al.*, 1999). The second pathway is mediated by mitochondria, which release cytochrome c, which can associate with Apaf-1 (apoptosis protease activating factor) and pro-caspase-9, triggering the activation of caspase-3 and apoptosis (Kromer *et al.*, 1997). Synthetic caspase inhibitors have been shown to be cardioprotective in our previous study (chapter 4) and in other studies (Haung *et al.*, 2000, Mocanu *et al.*, 2000); however the precise mechanism behind their cardioprotection is not yet clear. The caspase inhibitor (ZVAD) also failed to inhibit cytochrome c release from mitochondria using primary neonatal rat cardiomyocytes culture model, which suggests that cytochrome c release is upstream and independent of caspase activation (Bialik *et al.*, 1999). Therefore, research indicates that caspase inhibitors mediated cardioprotection is not dependent on mitochondrial cytochrome c pathway. An alternative mechanism operating at the level of cytosolic caspase activation can be suggested. The fact that both the broad and the specific caspase inhibitors were effective to approximately the same degree, in spite of the fact that the inhibitors were

reported to exert specific and selective anti-caspase effects, raise a question about the specificity of these drugs and the mechanism behind their cardioprotection. This again raised the possibility that they might share the simillar pathways.

There is abundant evidence in the literature suggesting that activation of PI3-Akt signalling pathway is a powerful survival signaling pathway leading to anti-apoptosis and anti-necrosis and cell survival (Wu *et al*., 2000, Yin *et al*., 2004, Hausenloy *et al.*, 2006). Cardioprotection conferred by postconditioing and preconditioning has been found to be mediated through activation of PI3-Akt survival signaling pathway (Hausenloy *et al.*, 2006). Furthermore, cadioprotection by pharmacological agents that mimic preconditioning effect such as bradykinin (Baxter & Ebrahim 2002), AMP579 (Strickler *et al.*, 1996) and drugs applied during the early phase of reperfusion such as Insulin (Jonassen *et al.*, 2001), Urocortin (Brar *et al.*, 2000, Schulman *et al.*, 2002), Bradykinin (Li & Sato 2001) and adenosine agonists (Schulte & Fredholm 2000), were also mediated through activation of PI3-Akt cell signalling pathway. Therefore, it appeared that activation of PI3-Akt survival kinase pathway may constitute a common survival pathway sufficient to induce a cardio-protective response during early reperfusion phase (Hausenloy & Yellon, 2004). Caspase inhibitors showed a promising cardioprotection when applied during early phase of reperfusion, which could also be mediated by activation of PI3-Akt pathway.

The aim of this study was to investigate whether cardioprotection by administration of the broad spectrum caspase inhibitor (ZVAD, 0.1µM and 25µM) or specific caspase-3 inhibitor

(DEVD, .07μM and 25μM) at different time points during reperfusion (at start of reperfusion, 15 min and 30 minutes after starting reperfusion) is mediated via PI3-Akt cell signalling pathway using PI3-Akt specific inhibitor (Wortmannin, 100nM).

5.2 Methods

5.2.1 Langendorff heart reperfusion model (chapter 3 methods, section 3.4)

Isolated rat hearts were subjected to 20 min stabilisation, and then underwent 35 min regional ischemia followed by 120 min reperfusion using Langendorff perfusion system. Caspase inhibitors (Broad spectrum caspase inhibitor, ZVAD, 0.1 µM and Specific caspase-3 inhibitor, DEVD, 0.07 µM) were added at the start of reperfusion, 15 minutes and 30 minutes after starting reperfusion in the presence and absence of PI3-Akt specific inhibitor Wortmannin (WORT, 100nM). At the end of the experimental protocol, infarction size to risk area ratio (I/R %) determined using TTC staining.

5.2.3 Analysis of isolated adult rat ventricular myocytes using flow cytometry (chapter 3 methods, section 3.6)

Isolated adult rat ventricular myocytes underwent 6 hours of hypoxia followed by 18 hours of reoxygenation. Caspase inhibitors (ZVAD 25µM, DEVD 25µM) were added at the start of reoxygenation, 15 minutes and 30 minutes after starting reoxygenation in the presence and absence of PI3-Akt specific inhibitor Wortmannin (WORT, 100nM). At the end of the experimental protocol, apoptotsis, necrosis, viability and intracellular caspase-3 activity were measured using flow cytometry analysis.

5.2.3 Western blot analysis (chapter 3 methods, section 3.5)

Western blot analysis was used to determine the protein expression level of cytosolic cytochrome c in hearts treated with ZVAD (0.1µM) and DEVD (0.07µM) administerd at start of reperfusion, 15 minutes, 30 minutes and 60 minutes after starting reperfusion. Western blot analysis was also used to determine the protein expression level of phospho-

Akt in hearts treated with ZVAD (0.1μM) and DEVD (0.07μM) in the presence and absence of PI3-Akt specific inhibitor Wortmannin (WORT, 100nM). A time course of administration with ZVAD and DEVD (5, 10, 20 and 120 minutes reperfusion) and analagous control ischemic-reperfused hearts was performed. Hearts treated with WORT were administered for only 10 minutes of reperfusion. Densitometry was measured using Quantity-One software.The mean density of control ischemic/reperfused hearts were compared to heart groups reperfused with ZVAD or DEVD ± WORT.

5.3 Results

5.3.1 Caspase inhibitors cardioprotection during early reperfusion phase is mediated via PI3-Akt kinase pathway.

5.3.1.1 Myocardial infarct size to risk area ratio (I/R %)

5.3.1.1.1 The cardioprotective effect of the broad spectrum caspase inhibitor (ZVAD, 0.1μM) when added at the start of reperfusion is blocked by PI3-Akt inhibitor, Wortmannin (WORT, 100nM)

In the present study, we aimed at assessing whether administration of PI3-Akt specific inhibitor Wortmannin (WORT, 100nM) would abolish the protection offered by the broad spectrum caspase inhibitor (ZVAD, 0.1μM) thus suggesting that the cardioprotective effects of ZVAD was via PI3-Akt pathway. There was a significant reduction in infarction/risk ratio (I/R %) with ZVAD treatment at the start of reperfusion throughout when compared to ischemic controls (ZVAD at reperfusion 17.3± 2, *vs* Control 54.3± 6.6, Tukey's post-hoc test $p<0.01$) Figure 5.1. To test whether ZVAD mediated cardioprotection was via PI3-Akt kinase pathway, PI3-Akt inhibitor (WORT), was added at the onset of reperfusion simultaneously with ZVAD. WORT blocked ZVAD cardioprotection, showing a significant increase in I/R% compared to ZVAD treated group (WORT+ZVAD 50.8± 6.2 *vs* ZVAD at reperfusion 17.3± 2 Tukey's post-hoc test $p<0.01$) Figure 5.1. Adding WORT alone at the start of reperfusion had no significant effect on I/R% compared to control (WORT 50 ± 4.4% *vs* Control 54.3 ± 6.6, Tukey's post-hoc test $p>0.05$) Figure 5.1. Overall, there was a significant reduction in I/R% with ZVAD reperfused hearts in comparison to ischemic/reperfusion control hearts which was blocked by PI3-Akt specific inhibitor WORT.

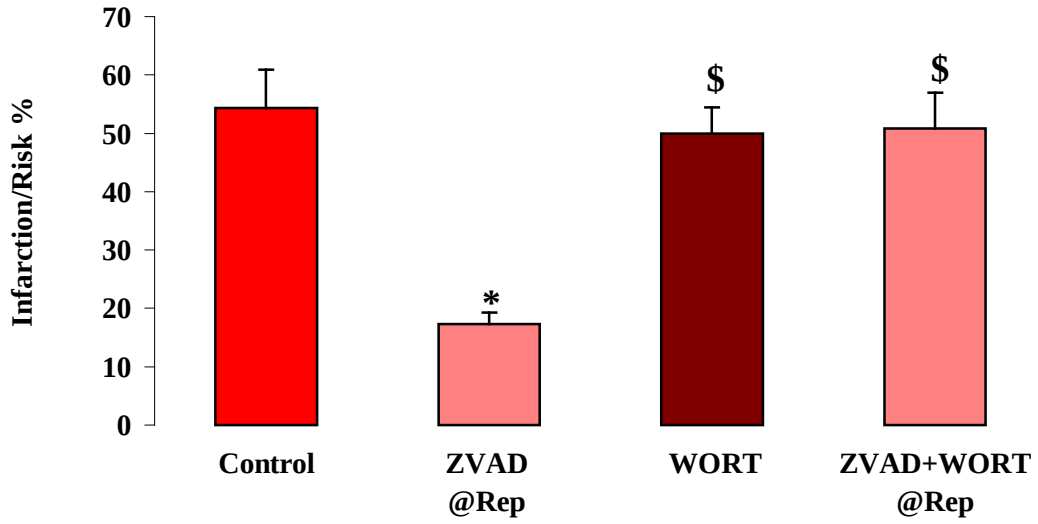


Figure 5.1: Infarct size to Risk ratio (%) in control and broad spectrum caspase inhibitor (ZVAD, 0.1µM) treated hearts. Isolated perfused rat hearts subjected to 35 minutes of ischemia and 120 minutes of reperfusion where the ZVAD was administered at the start of reperfusion period throughout in the presence and absence of the of PI3-Akt inhibitor; Wortmannin (WORT, 100n M). *P<0.01 *vs*. Control $P<0.01 *vs* ZVAD. Results are shown as mean + SEM, n=5.

5.3.1.1.2 The cardioprotective effect of the specific caspase-3 inhibitor (DEVD, 0.07µM) when added at the start of reperfusion is blocked by PI3-Akt inhibitor, Wortmannin (WORT, 100nM)

Treatment with specific caspase-3 inhibitor (DEVD, 0.07µM) immediately after starting reperfusion throughout resulted in significant cardioprotection when compared to ischemic control group (14.38 ± 3.87 % *vs* 54.25 ± 6.60 % control, Tukey's post-hoc test p<0.01) Figure 5.2. This cardioprotection was significantly reduced by PI3-Akt inhibitor; Wortmannin (WORT, 100nM) when co-administered with DEVD at the start of reperfusion throughout, showing a significant increase in I/R% compared to DEVD treated group (DEVD+WORT 41.0 ± 5.5 % *vs* 14.38 ± 3.87 % EDVD at reperfusion, Tukey's post-hoc test p<0.01) Figure 5.2. Overall, there was a significant reduction in I/R% with DEVD

reperfused hearts in comparison to ischemic/reperfusion control hearts which was blocked by PI3-Akt specific inhibitor WORT.

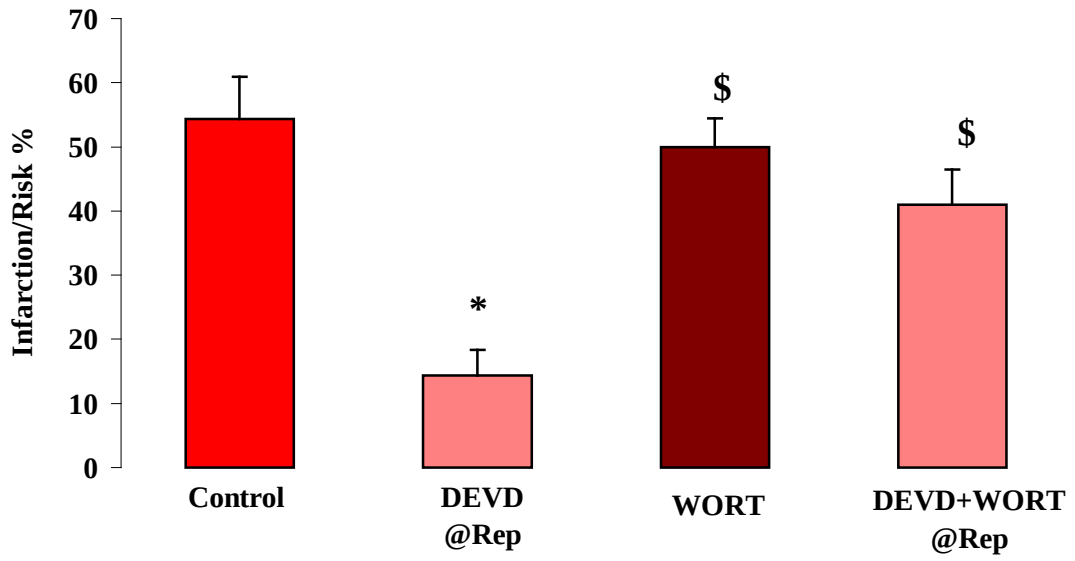


Figure 5.2: Infarct size to Risk ratio (%) in control and specific caspase-3 inhibitor (DEVD) treated hearts. Isolated perfused rat hearts subjected to 35 minutes of ischemia and 120 minutes of reperfusion. The DEVD (0.07µM) was administered at the start of reperfusion period throughout in the presence and absence of the PI3-Akt inhibitor; Wortmannin (WORT, 100nM). *P<0.01 *vs*. Control $P<0.01 *vs* DEVD. Results are shown as mean + SEM, n=5.

5.3.1.2 Analysis of isolated adult rat ventricular myocytes using flow cytometry.

5.3.1.2.1 The anti-apoptotic effect of the broad spectrum caspase inhibitor (ZVAD, 25µM) when added at the start of reoxygenation is blocked by PI3-Akt inhibitor, Wortmannin (WORT, nM)

Isolated cardiomyocytes underwent 6 hours of hypoxia in 5% CO_2 0% O_2 followed by 18 hours of reoxygenation. Broad spectrum caspase inhibitor (ZVAD, 25µM) was administered throughout reoxygenation in the presence and absence of PI3-Akt inhibitor, Wortmannin (WORT, 100nM). Mean % apoptotic myocytel populations was significantly decreased with ZVAD treatment administered at the start of reoxygenation throughout in comparison to control hypoxic / reoxgenenated myocytes (ZVAD at Reox 22.90 ± 4.77 %

vs Hypox/Reox 48.25 ± 3.70 %, Tukey's post-hoc test P<0.01) Figure 5.3. Treatment with PI3-Akt inhibitor (WORT) simultaneously with ZVAD abolished the anti-apoptotic cardioprotection afforded by ZVAD, showing a significant increase in % apoptotic myocytes compared to ZVAD treated myocytes (WORT+ZVAD at Reox 40.02 ± 6.2 % *vs* ZVAD at Reox 22.90 ± 4.77, Tukey's post-hoc test p<0.05) Figure 5.3. % Apoptotic myocytes populations in treatment with WORT alone were comparable to control treated myocytes (Hypox/Reox 48.25 ± 3.70 *vs* WORT: 40.79 ± 5.136, Tukey's post-hoc test p> 0.05) Figure 5.3. Overall, there was a significant reduction in % apoptotic myocytes populations with ZVAD treated myocytes in comparison to control hypoxia/reoxygenation myocytes which was blocked by PI3-Akt specific inhibitor WORT.

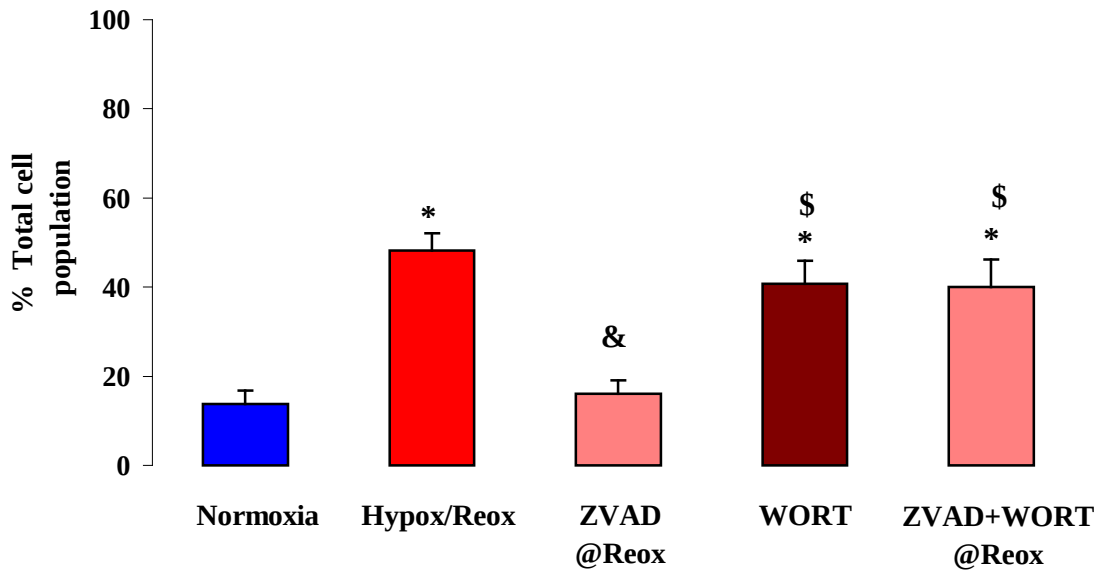


Figure 5.3: Assessment of apoptosis in isolated adult rat cardiomyocytes subjected to 6 hours hypoxia and 18 hours of reoxygenation. The broad spectrum caspase inhibitor (ZVAD, 25µM) was added at the onset of reoxygenation throughout in the presence and absence of the of PI3-Akt inhibitor; Wortmannin (WORT, 100nM). Results are shown as mean + SEM and are expressed as a percentage of the total cells counted.* p<0.05 *vs*. Normoxia, & p<0.05 *vs*. Hypox/Reox Control $ p<0.05 *vs*. ZVAD at Reox, n=5.

5.3.1.2.2 The cytoprotective effect of the broad spectrum caspase inhibitor (ZVAD, 25µM) when added at the start of reoxygenation is blocked by PI3-Akt inhibitor, Wortmannin (WORT, 100nM)

Mean % viable myocytes populations was significantly increased with broad spectrum caspase inhibitor (ZVAD, 25µM) treatment administered at the start of reoxygenation throughout in comparison to control hypoxic / reoxgenenated myocytes (ZVAD at Reox 63.62 ± 7.68 % *vs* Hypox/Reox 18.25 ± 3.89 %, P<0.01) Figure 5.4. Co-administration of PI3-Akt inibitor (WORT, 100nM) with ZVAD at the start of reoxygenation throughout significantly abrogated the protection afforded by ZVAD (ZVAD+WORT 35.58 ± 10.22 % *vs* ZVAD at Reox 63.62 ± 7.68, p<0.05) Figure.5.4.

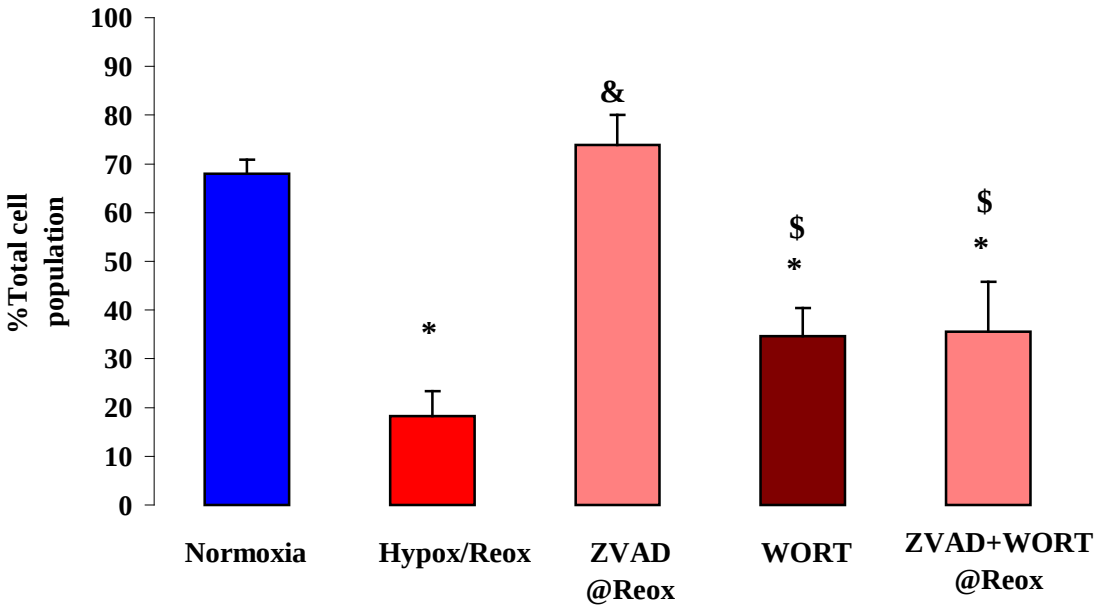


Figure 5.4: Assessment of cell viability in isolated adult rat cardiomyocytes subjected to 6 hours hypoxia and 18 hours of reoxygenation. The broad spectrum caspase inhibitor (ZVAD, 25µM) was added at the onset of reoxygenation in the presence and absence of the of PI3-Akt inhibitor; Wortmannin (WORT, 100n M). Results are shown as mean + SEM and are expressed as a percentage of the total cells counted.* p<0.05 *vs*. Normoxia, & p<0.05 *vs*. Hypox/Reox Control $ p<0.05 *vs*. ZVAD at Reox, n=5.

5.3.1.2.3 The anti-necrotic effect of the broad spectrum caspase inhibitor (ZVAD, 25µM) when added at the start of reoxygenation is blocked by PI3-Akt inhibitor, Wortmannin (WORT, 100nM)

Treatment with PI3-Akt inhibitor (WORT, 100 n M) and ZVAD (25µM) at the start of reoxygenation abolished the anti-necrotic effect afforded by ZVAD alone, showing a significant increase in % necrotic cells compared to ZVAD treated myocytes (ZVAD 10.0 ± 3.4 *vs*. WORT + ZVAD 24.4 ± 4.4, Tukey's post-hoc test $p<0.05$) Figure 5.5. Treatment with WORT alone was comparable to hypoxia/reoxygenation control treated myocytes (Hypox/Reox 33.5 ± 2.2 *vs*. WORT 24.5 ± 1.9, Tukey's post-hoc test $p>0.05$) Figure 5.5.

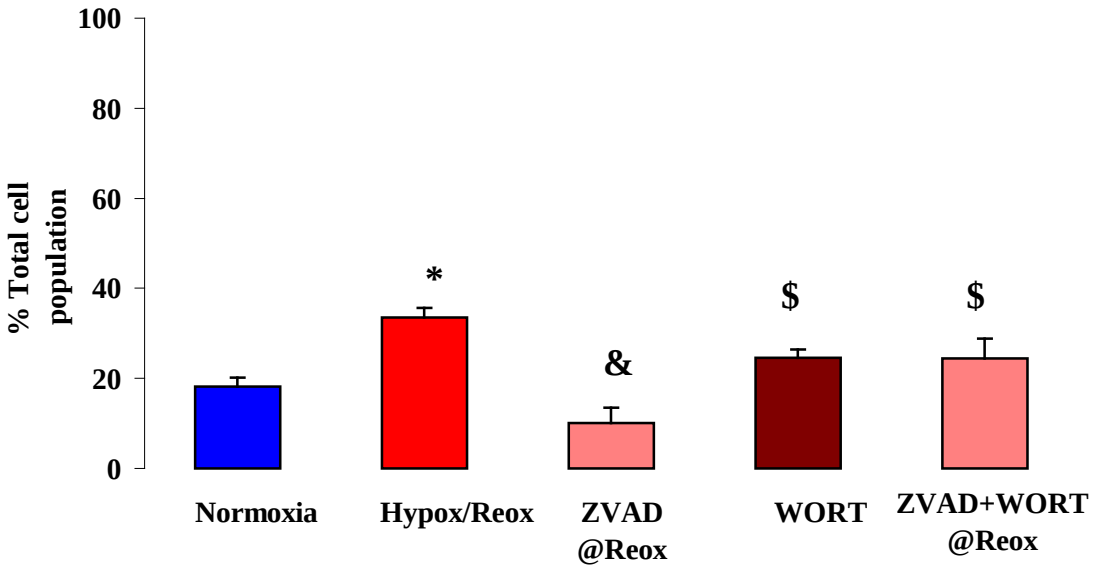


Figure 5.5: Assessment of necrosis in isolated adult rat cardiomyocytes subjected to 6 hours hypoxia and 18 hours of reoxygenation. The broad spectrum caspase inhibitor (ZVAD, 25µM) was added at the onset of reoxygenation throughout in the presence and absence of the of PI3-Akt inhibitor; Wortmannin (WORT, 100nM). Results are shown as mean + SEM and are expressed as a percentage of the total cells counted.* $p<0.05$ *vs*. Normoxia & $p<0.05$ vs. Hypox/Reox Control $ $p<0.05$ *vs*. ZVAD at Reox, n=5.

5.3.1.2.4 The inhibitory effect of the broad spectrum caspase inhibitor (ZVAD, 25μM) when added at the start of reoxygenation on caspase-3 activity level is blocked by PI3-Akt inhibitor, Wortmannin (WORT, 100nM)

To determine the role of the PI3K-Akt cell survival pathway on Caspase 3 activity level in broad spectrum caspase inhibitor (ZVAD, 25μM) mediated cardioprotection, isolated adult rat cardiac myocytes underwent 6 hours of hypoxia and 18 hours of reoxygenation where the ZVAD was administered in the presence and absence PI3-Akt inhibitor Wortmannin (100nM) throughout reoxygenation. Administration of ZVAD at reoxygenation significantly reduced caspase 3 activity compared to control non treated isolated adult rat cardiomyocytes subjected to 6 hours of hypoxia and 18 hours of reoxygenation (ZVAD at Reox 141.92 ± 26.63 % *vs* Hypo/Reox 375.47 ± 90.88%, Tukey's post-hoc test $p<0.05$) Figure 5.6. Administration of the PI3-Akt inhibitor (WORT, 100nM) with the ZVAD at reoxygenation blocked the decrease in caspase 3 activity afforded by ZVAD when administered alone at reoxygenation (ZVAD+WORT at Reox 248.89 ± 37.85 % *vs* ZVAD at Reox 141.92 ± 26.63 %, Tukey's post-hoc test $P<0.05$) Figure 5.6. WORT when administered alone had no significant effect on caspase 3 activity level compared to the control hypoxia/eoxygenation myocytes (WORT 308.0 ± 37.85 % *vs* Hypo/Reox 375.5 ± 90.88%, Tukey's post-hoc test $p>0.05$) Figure 5.6.

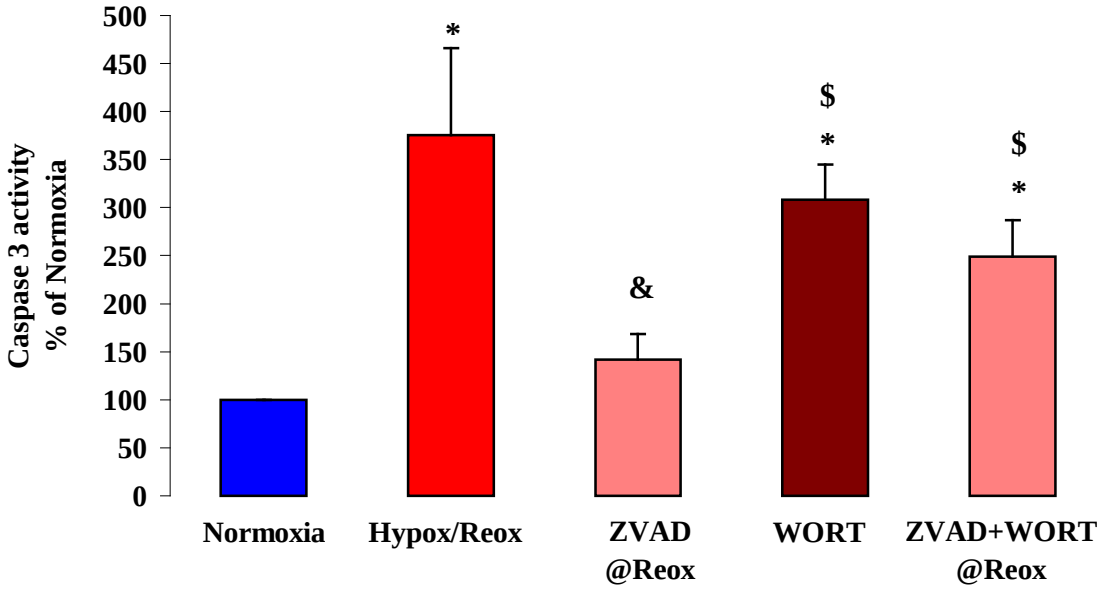


Figure 5.6: Caspase 3 activity in isolated adult rat cardiac myocytes subjected to 6 hours of hypoxia followed by 18 hours of reoxygenation. The broad spectrum caspase inhibitor (ZVAD, 25µM) was administered at the onset of reoxygenation in the presence and absence of the of PI3-Akt inhibitor; Wortmannin (WORT, 100nM). Results are shown as mean + SEM and are expressed as a percentage of the normoxia control values. * p<0.05 *vs*. Normoxia & p<0.05 *vs*. Hypox/Reox Control $ p<0.05 vs. ZVAD at Reox, n=5.

5.3.1.2.5 The anti-apoptotic effect of the specific caspase-3 inhibitor (DEVD, 25µM) when added at the start of reoxygenation is blocked by PI3-Akt inhibitor, Wortmannin (WORT, 100nM)

Treatment with specific caspase-3 inhibitor (DEVD, 25µM) at the start of reoxygenation throughout resulted in a significant decrease in % apoptotic myocyte population when compared to hypoxia/reoxygenation control myocytes (DEVD at Reox 22.39 ± 5.94%. *vs* Hypo/Reox 48.24 ± 3.70 %, Tukey's post-hoc test $p<0.05$) Figure 5.7. Results showed that PI3-Akt inhibitor; wortmannin (WORT, 100nM) abolished anti-apoptotic effect afforded by DEVD when added simultaneously at the start of the reoxygenation throughout (DEVD+WORT at Reox 40.79 ± 5.13 *vs* DEVD at Reox 22.39 ± 5.94%, Tukey's post-hoc test $p< 0.05$) Figure 5.7.

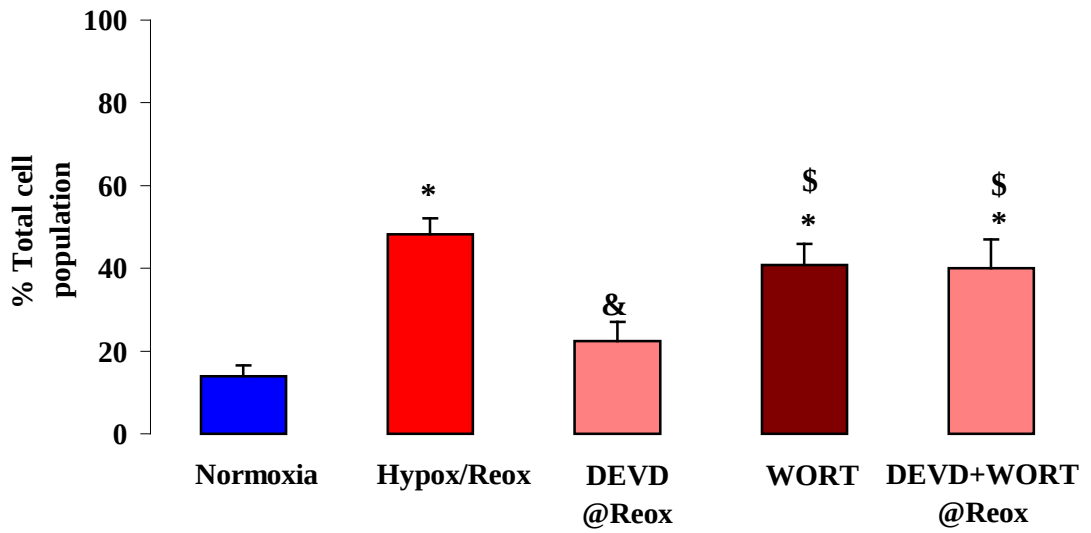


Figure 5.7: Assessment of apoptosis in isolated adult rat cardiomyocytes subjected to 6 hours hypoxia and 18 hours of reoxygenation. The specific caspase-3 inhibitor (DEVD, 25µM) was added at the onset of reoxygenation in the presence and absence of the of PI3-Akt inhibitor; Wortmannin (WORT, 100nM). Results are shown as mean + SEM and are expressed as a percentage of the total cells counted.* p<0.05 *vs*. Normoxia & p<0.05 *vs*. Hypox/Reox Control $ p<0.05 *vs*. DEVD at Reox, n=5.

5.3.1.2.6 The cytoprotective effect of the specific caspase-3 inhibitor (DEVD, 25µM) when added at the start of reoxygenation is blocked by PI3-Akt inhibitor, Wortmannin (WORT, 100nM)

Mean % viable cell populations were significantly increased with DEVD treatment in comparison to control hypoxic/reoxgenenated myocytes (Hypox/Reox 18.25 ± 3.89 % *vs*. DEVD at Reox 61.54 ± 3.89 %, Tukey's post-hoc test p<0.05) Figure 5.8. Treatment with PI3-Akt inhibitor (WORT, 100nM) abolished the protection afforded by DEVD showing a significant decrease in % viable cells compared to DEVD treated myocytes (DEVD at Reox 61.54 ± 3.89 % *vs* DEVD+WORT 34.72 ± 8.50, Tukey's post-hoc test p<0.05) Figure 5.8.

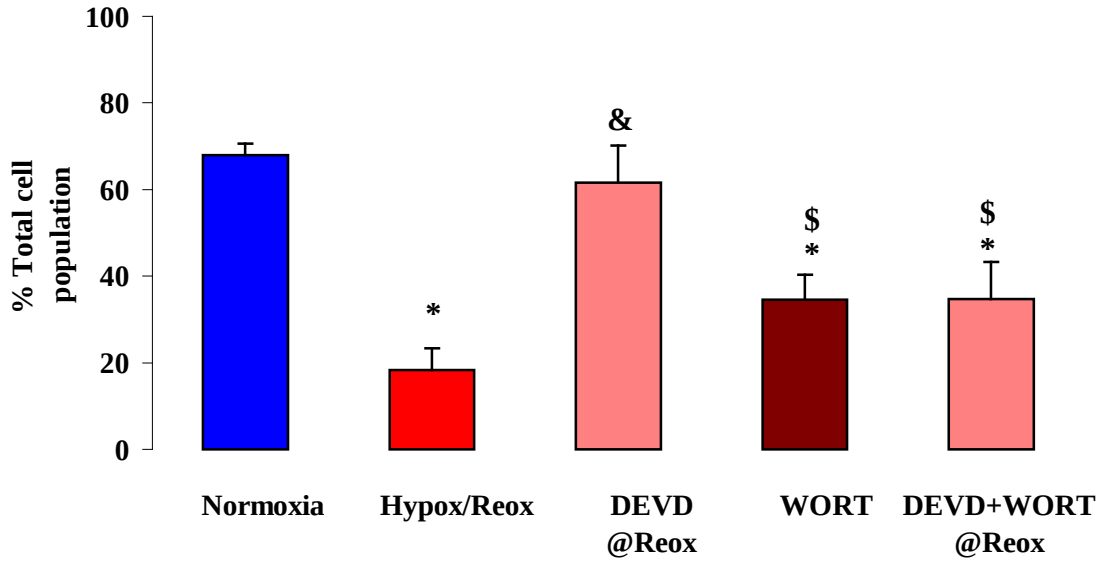


Figure 5.8: Assessment of cells viability in isolated adult rat cardiomyocytes subjected to 6 hours hypoxia and 18 hours of reoxygenation. The specific caspase-3 inhibitor (DEVD, 25µM) was added at the onset of reoxygenation in the presence and absence of the of PI3-Akt inhibitor; Wortmannin (WORT, 100nM). Results are shown as mean + SEM and are expressed as a percentage of the total cells counted.* $p<0.05$ *vs.* Normoxia & $p<0.05$ *vs.* Hypox/Reox Control $ $p<0.05$ *vs.* DEVD at Reox, n=5.

5.3.1.2.7 The anti-necrotic effect of the specific caspase-3 inhibitor (DEVD, 25µM) when added at the start of reoxygenation is blocked by PI3-Akt inhibitor, Wortmannin (WORT, 100nM)

Administration of the specific-caspase-3 inhibitor (DEVD, 25µM) at the start of reoxygenation throughout significantly decreased the necrotic cell population when compared to control Hypoxia/reoxygenated myocytes (16.0 ± 5.2 % *vs.* 33.5 ± 2.1 % Hyp/Reox, Tukey's post-hoc test $p<0.05$) Figure 5.9. This anti-necrotic effect of DEVD was abolished in the presence of the PI3-Akt inhibitor (WORT, 100nM) (25.2 ± 2.0 % *vs* 16.0 ± 2.1 DEVD at Reox, Tukey's post-hoc test $p<0.05$) Figure 5.9. The PI3-Akt inhibitor alone had no significant effect on necrotic cell populaton when compared to control hypoxia/reoxenated myocytes (24.5 ± 4.2 vs 33.5 ± 2.1 Hypox/Reox, Tukey's post-hoc test $p>0.05$) Figure 5.9.

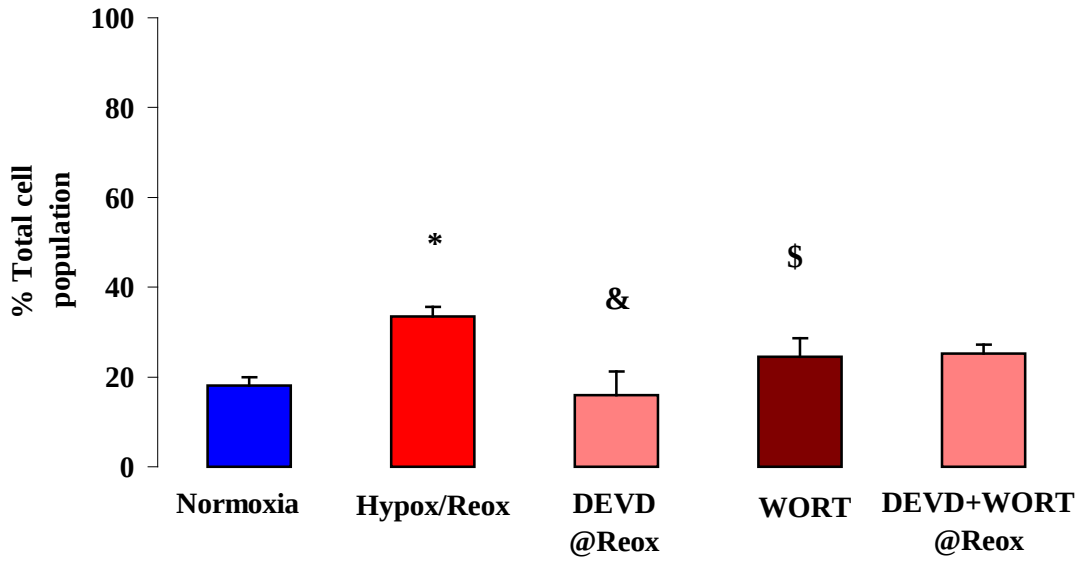


Figure 5.9: Assessment of necrosis in isolated adult rat cardiomyocytes subjected to 6 hours hypoxia and 18 hours of reoxygenation. The specific caspase-3 inhibitor (DEVD, 25µM) was added at the onset of reoxygenation in the presence and absence of the of PI3-Akt inhibitor; Wortmannin (WORT, 100nM). Results are shown as mean + SEM and are expressed as a percentage of the total cells counted.* $p<0.05$ *vs*. Normoxia & $p<0.05$ *vs*. Hypox/Reox Control $ $p<0.05$ *vs*. DEVD at Reox. n=5

5.3.1.2.8 The inhibitory effect of the specific caspase3 inhibitor (DEVD, 25µM) when added at the start of reoxygenation on caspase-3 activity is blocked by PI3-Akt inhibitor, Wortmannin (WORT, 100nM)

Administration of the specific caspase-3 inhibitor (DEVD, 25µM) at reoxygenation throughout significantly reduced caspase 3 activity compared to non treated isolated adult rat cardiomyocytes subjected to 6 hours of hypoxia and 18 hours of reoxygenation (127.5 ± 21.2 % *vs* Hypo/Reox 375.5 ± 91.0%, Tukey's post-hoc test $p<0.05$) Figure 5.10. Administration of the PI3-Akt inhibitor (WORT, 100nM) with the DEVD at reoxygenation blocked the decrease in caspase 3 activity afforded by DEVD when administered at reoxygenation (242.4 ± 19.7 % *vs* DEVD at Reox 127.5 ± 21.2 %, Tukey's post-hoc test $P<0.05$) Figure 5.10. WORT when administered alone had no significant effect on caspase

3 activity level compared to the hypoxia/reoxygenated control myocytes (WORT 308.1 ± 37.8 % *vs* Hypo/Reox 375.5 ± 91.0 %, Tukey's post-hoc test p>0.05) Figure 5.10.

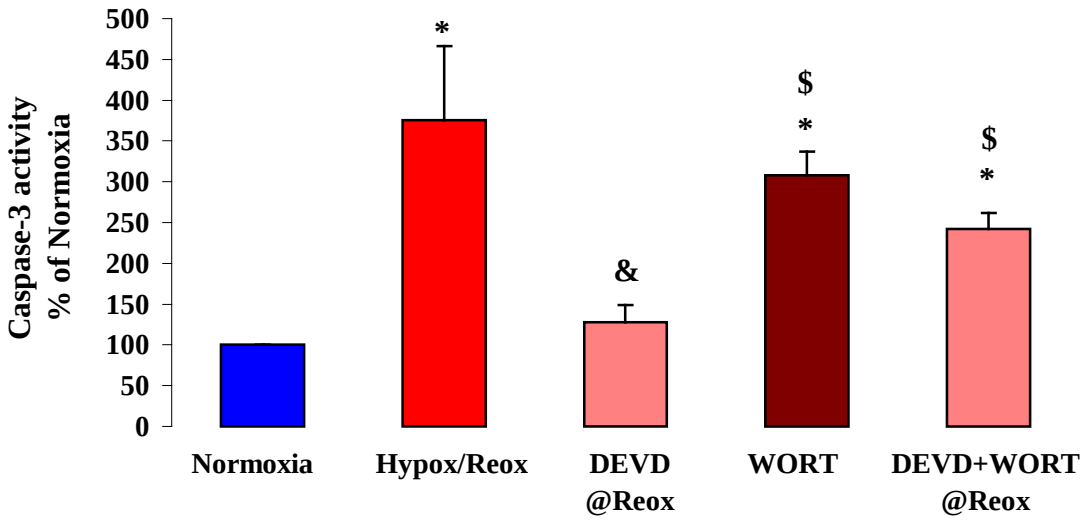


Figure 5.10: Caspase 3 activity in isolated adult rat cardiac myocytes subjected to 6 hours of hypoxia followed by 18 hours of reoxygenation. The specific caspase-3 inhibitor (DEVD, 25µM) was administered at the onset of reoxygenation in the presence and absence of the of PI3-Akt inhibitor; Wortmannin (WORT, 100nM). Results are shown as mean + SEM and are expressed as a percentage of the normoxia control values. * p<0.05 *vs*. Normoxia & p<0.05 *vs*. Hypox/Reox Control $ p<0.05 *vs*. DEVD at Reox, n=5.

5.3.1.2.9 Results summary

In summary both the broad spectrum caspase inhibitor (ZVAD) and the specific caspase 3 inhibitor (DEVD) resulted in significant cardioprotection by siginicant reduction in infarction size within the risk area, significant reduction in apoptotic and necrotic cellular death and siginificant reduction in intracellular caspase 3 acitivity when administered immediately after starting reperfusion. This cardioprotection was blocked by co administration of PI3-Akt inhibitor (WORT, 100nM) as shown in table 5.1.

Group	I/R %	Viability	Apoptosis	Necrosis	Caspase 3 activity (%of Normoxia)
Normoxia		68.0± 2.9	13.8± 3.0	18.2 ±2.0	100%
Control Ischemia/Rep	54.2±6.6	18.2±5.1	48.2± .8	33.5± 2.2	375.5±90.8
ZVAD@Rep	17.3±2.0*	73.9±6.2*	16.1±3.0*	10.0±3.4*	141.9±26.6*
DEVD@Rep	14.4±3.9*	61.5±8.6*	22.4±4.6*	16.0±5.2*	127.5±21.2*
WORT	50.0±4.4	34.6±5.8	41.0±5.1	24.5 ±1.9	308.0 ±37.8
ZVAD+WORT@Rep	50.8±6.2	35.6±10.2	40.0±6.2	24.4±4.4	249.0± 37.8
DEVD+WORT@Rep	41.0±5.5	34.7±8.5	41.0±5.1	25.2±2.0	242.4±19.7

Table 5.1 Summary table showing the effect of the broad spectrum caspase inhibitor (ZVAD) and the specific caspase 3 inhibitor (DEVD) on I/R%, cellular viability, apoptosis, necrosis and intracellular caspase 3 activity when admisitered at the start of reperfusion in the presence and absence of the of PI3-Akt inhibitor; Wortmannin (WORT, 100nM). Results are shown as mean ± SEM * p<0.05 *vs*. control ischemia reperfusion.

5.3.2 Caspase inhibitors cardioprotection during late phase of reperfusion is not mediated via PI3-Akt kinase pathway.

5.3.2.1 Myocardial infarct size to risk area ratio (I/R %)

5.3.2.1.1 The cardioprotective effect of the broad spectrum caspase inhibitor (ZVAD, 0.1μM) when added at 15 minutes and 30 minutes after starting reperfusion is not blocked by PI3-Akt inhibitor, Wortmannin (WORT, 100nM)

As we demonstrated in chapter 4, delayed administraton of the broad spectrum caspase inhibitor (ZVAD, 0.1μM) 15 minutes and 30 minutes after starting reperfusion was still cardioprotective. In this study, we aimed at assessing whether administration of PI3-Akt specific inhibitor Wortmannin (WORT, 100nM) would abolish the protection offered by ZVAD thus suggesting that the cardioprotective effects of ZVAD at 15 minutes and 30 minutes after starting reperfusion was via PI3-Akt pathway. There was significant reduction in infarction/risk ratio (I/R %) with ZVAD administered at 15 minutes and 30 minutes after starting reperfusion when compared to ischemic controls hearts (ZVAD at 15 minutes 30.48 ± 3.41%, ZVAD at 30 minutes 31.60 ± 3.99% *vs* Control 54.3± 6.6, $p<0.01$) Figure 5.11. However, adding PI3-Akt inhibitor; Wortmannin (WORT) simultaneously with ZVAD at 15 minutes and 30 minutes after starting reperfusion did not block ZVAD mediated cardioprotection (WORT+ZVAD at 15 minutes 29.6± 4.7, WORT+ZVAD at 30 minutes 26.2 ± 6.6 *vs* Control 54.3± 6.6, $p<0.01$) Figure 5.11.

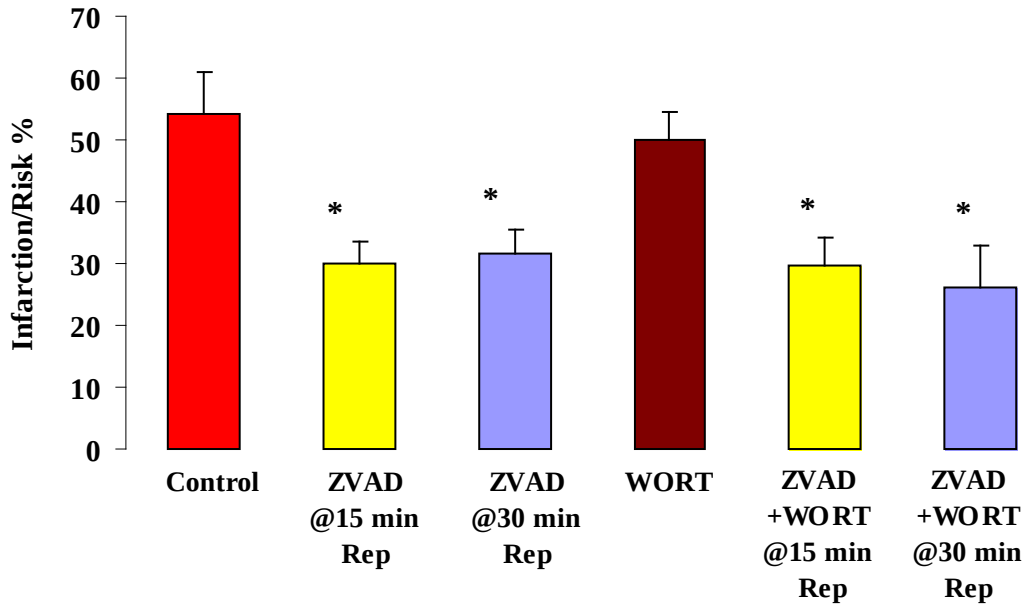


Figure 5.11: Infarct size to Risk ratio (I/R %) in control and broad spectrum caspase inhibitor (ZVAD, 25µM) treated hearts. Isolated perfused rat hearts subjected to 35 minutes of ischemia and 120 minutes of reperfusion where the ZVAD was administered at 15 and 30 minutes after starting reperfusion period in the presence and absence of the of PI3-Akt inhibitor; Wortmannin (WORT, 100nM). *P<0.01 *vs.* Control. Results are shown as mean + SEM, n=5.

5.3.2.1.2 The cardioprotective effect of the specific caspase-3 inhibitor (DEVD, 0.07µM) when added at 15 minutes and 30 minutes after starting reperfusion is not blocked by PI3-Akt inhibitor, Wortmannin (WORT, 100nM)

Treatment with specific caspase-3 inhibitor (DEVD, 0.07 µM) at 15 minutes and 30 minutes after starting reperfusion resulted in a significant decrease in I/R % (DEVD at 15 minutes 15.71 ± 3.59 %. DEVD at 30 minutes 18.51 ± 3.69 % *vs* Control 54.25 ± 6.60 % $p<0.01$) Figure 5.12. Results showed that PI3-Akt inhibitor; wortmannin (WORT, 100nM) did not block DEVD mediated cardioprotection when added simultaneously at 15 minutes and 30 minutes after starting reperfusion (DEVD+WORT at 15 minutes reperfusion 17.96

± 4.3 %, DEVD+WORT at 30 minutes reperfusion 18.9 ± 3.6 % *vs* Control 54.25 ± 6.60 % p<0.01) Figure 5.12.

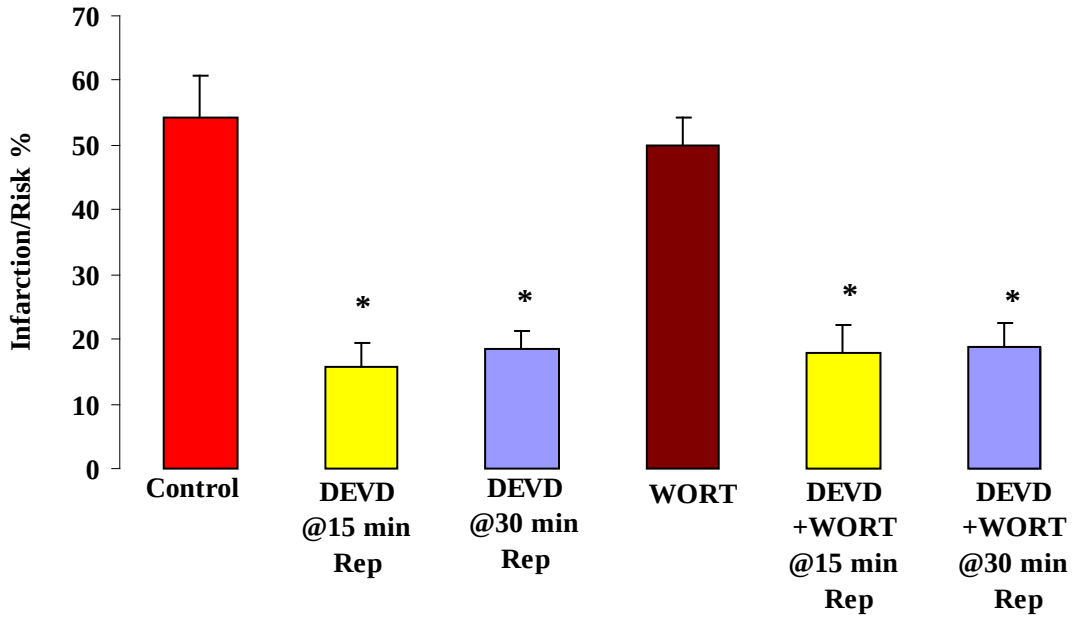


Figure 5.12: Infarct size to Risk ratio (I/R %) in control and specific caspase-3 inhibitor (DEVD, 0.07µM) treated hearts. Isolated perfused rat hearts subjected to 35 minutes of ischemia and 120 minutes of reperfusion.The DEVD was administered at 15 and 30 minutes after starting reperfusion period in the presence and absence of the of PI3-Akt inhibitor; Wortmannin (WORT, 100nM). *P<0.01 *vs*. Control Results are shown as mean + SEM, n=5.

5.3.2.2 Analysis of isolated adult rat ventricular myocytes using flow cytometry.

5.3.2.2.1 The anti-apoptotic effect of the broad spectrum caspase inhibitor (ZVAD, 25µM) when added at 15 minutes and 30 minutes after starting reoxygenation is not blocked by PI3-Akt inhibitor, Wortmannin (WORT; 100nM)

Delayed administration of the broad spectrum caspase inhibitor (ZVAD, 25µM) 15 minutes and 30 minutes post reoxygenation significantly decreased the % apoptotic myocytes when compared to contol Hypox/Reox myocytes (26.8 ± 1.7 %, 28.5 ± 5.1 % respectively *vs.* 48.2 ± 3.8 % Hyp/Reox, Tukey's post-hoc test, p<0.01) Figure 5.13. This anti-apoptotic effect by ZVAD at 15 minutes and 30 minutes was not affected by the PI3-Akt specific

inhibitor Wortmannin (WORT, 100nM) (23.7 ± 4.6 %, 20.3 ± 4.2 % respectively *vs*. 48.2 ± 3.8 % Hyp/Reox, Tukey's post-hoc test, p<0.01) Figure 5.13

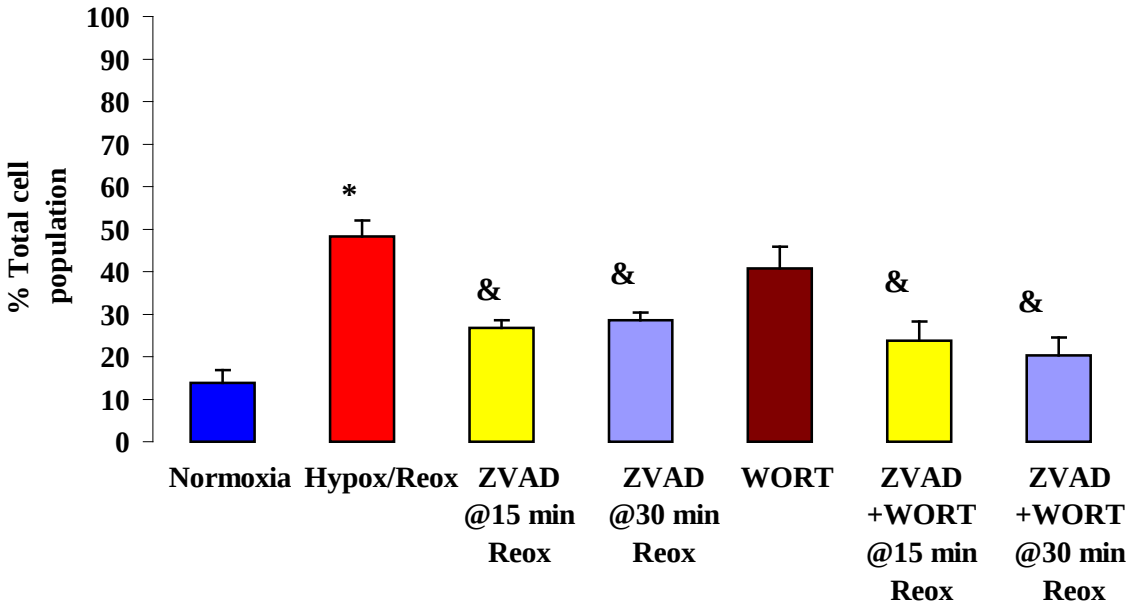


Figure 5.13: Assessment of apoptosis in isolated adult rat cardiomyocytes subjected to 6 hours hypoxia and 18 hours of reoxygenation. The broad spectrum caspase inhibitor (ZVAD, 25µM) was added at 15 minutes and 30 minutes after starting reoxygenation in the presence and absence of the of PI3-Akt inhibitor; Wortmannin (WORT, 100nM). Results are shown as mean + SEM and are expressed as a percentage of the total cells counted.* p<0.05 *vs*. Normoxia & p<0.05 *vs*. Hypox/Reox Control, n=5.

5.3.2.2.2 The cytoprotective effect of the broad spectrum caspase inhibitor (ZVAD, 25µM) when added at 15 minutes and 30 minutes after starting repxygenation is not blocked by PI3-Akt inhibitor, Wortmannin (WORT/100nM)

Delayed administration of (ZVAD, 25µM) 15 minutes and 30 minutes post reoxygenation significantly increased myocytes viabilty when compared to contol Hypox/Reox myocyte (42.1 ± 5.1 %, 47.0 ± 3.6 % respectively *vs*. 18.2 ± 5.1 % Hyp/Reox, Tukey's post-hoc test, p<0.01) Figure 5.14. This cytoprotective effect by ZVAD at 15 minutes and 30 minutes was not abolished by the PI3-Akt specific inhibitor Wortmannin (WORT, 100nM) (47.0 ±

4.3 %, 48.0 ± 2.2 % respectively *vs.* 18.2 ± 5.1 % Hyp/Reox, Tukey's post-hoc test, p<0.01), Figure 5.14.

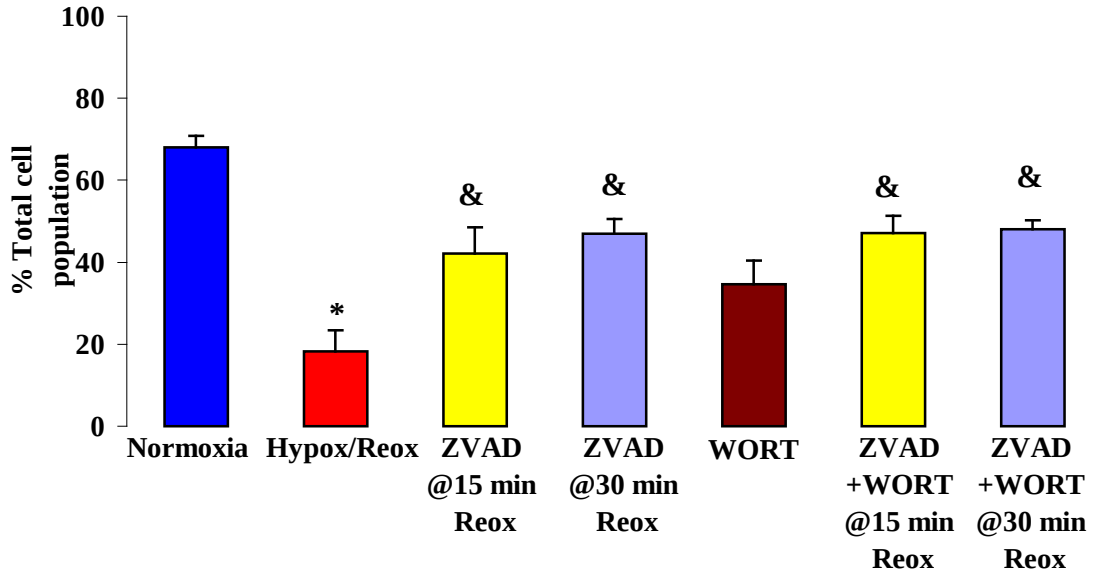


Figure 5.14: Assessment of myocytes viabilty in isolated adult rat cardiomyocytes subjected to 6 hours hypoxia and 18 hours of reoxygenation. The broad spectrum caspase inhibitor (ZVAD, 25µM) was added at 15 minutes and 30 minutes after starting reoxygenation in the presence and absence of the of PI3-Akt inhibitor; Wortmannin (WORT, 100nM). Results are shown as mean + SEM and are expressed as a percentage of the total cells counted.* p<0.05 *vs*. Normoxia, & p<0.05 *vs*. Hypox/Reox Control, n=5.

5.3.2.2.3 The inhibitory effect of the broad spectrum caspase inhibitor (ZVAD, 25µM) when added at 15 minutes and 30 minutes after starting reoxygenation on caspase-3 level is not blocked by PI3-Akt inhibitor, Wortmannin (WORT, 100nM)

Results showed that there is a significant reduction in caspase-3 activity level after treatment with the broad spectrum caspase inhibitor (ZVAD, 25µM) administered at 15 minutes and 30 minutes after starting reperfusion when compared to control myocytes exposed to hypoxia and reoxgenation conditions (ZVAD at 15 minutes 123.84 ± 13.70 %, ZVAD at 30 minutes 138.5 ± 29.58 % *vs* Hypox/Reox 375.5 ± 90.88 %, p<0.05) Figure 5.15. However, adding PI3-Akt inhibitor; Wortmannin (WORT, 100nM) simultaneously

with ZVAD at 15 minutes and 30 minutes after starting reoxygenation did not block ZVAD mediated reduction in caspase 3 activity (WORT+ZVAD at 15 minutes Reox 185.2 ± 26.8 %, WORT+ZVAD at 30 minutes Reox 146.5 ± 35.26 % *vs* Hypox/Reox 375.5 ± 90.88 %, $p<0.05$) Figure 5.15.

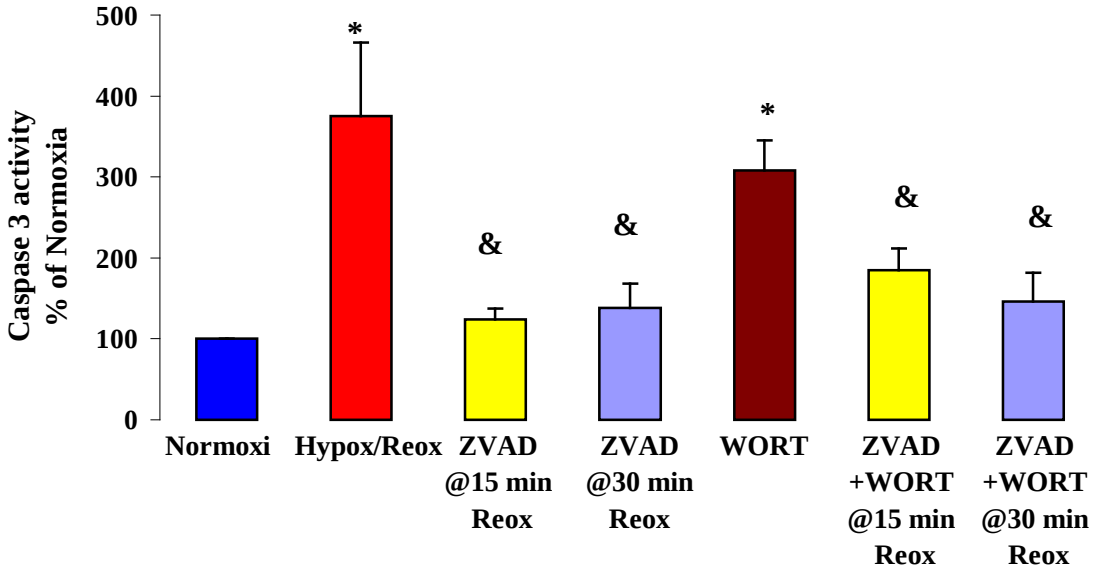


Figure 5.15: Caspase 3 activity in isolated adult rat cardiac myocytes subjected to 6 hours of hypoxia followed by 18 hours of reoxygenation. The broad spectrum caspase inhibitor (ZVAD, 25µM) was administered at 15 minutes and 30 minutes after starting reoxygenation in the presence and absence of the of PI3-Akt inhibitor; Wortmannin (WORT, 100nM). Results are shown as mean + SEM and are expressed as a percentage of the normoxia control values. * $p<0.05$ *vs*. Normoxia, & $p<0.05$ *vs*. Hypox/Reox Control, n=5.

5.3.2.2.4 The anti-apoptotic effect of the specific caspase-3 inhibitor (DEVD, 25µM) when added at 15 minutes and 30 minutes after starting repxygenation is not blocked by PI3-Akt inhibitor, Wortmannin (WORT, 100nM)

Treatment with PI3-Akt inhibitor (WORT, 100nM) and DEVD (25µM) simultaneously at 15 minutes and 30 minutes after starting reoxygenation did not affect the anti-apoptotic effect afforded by DEVD alone, showing a significant decrease in % apoptotic myocytescells compared to DEVD treated myocytess at 15 minutes and 30 minutes (22.8 ±

7.4 %, 26.5 ± 5.0 % respectively *vs*. 19.0 ± 5.2 % DEVD at 30 min Reox and DEVD at 30 minutes Reox 28.8 ± 5.5 %, Tukey's post-hoc test p>0.05) Figure 5.16. Treatment with WORT alone was comparable to Hypox/Reox control treated cells (Hypox/Reox 33.5 ± 2.2 % *vs*. WORT 24.5 ± 1.9 %, Tukey's post-hoc test p>0.05) Figure 5.16.

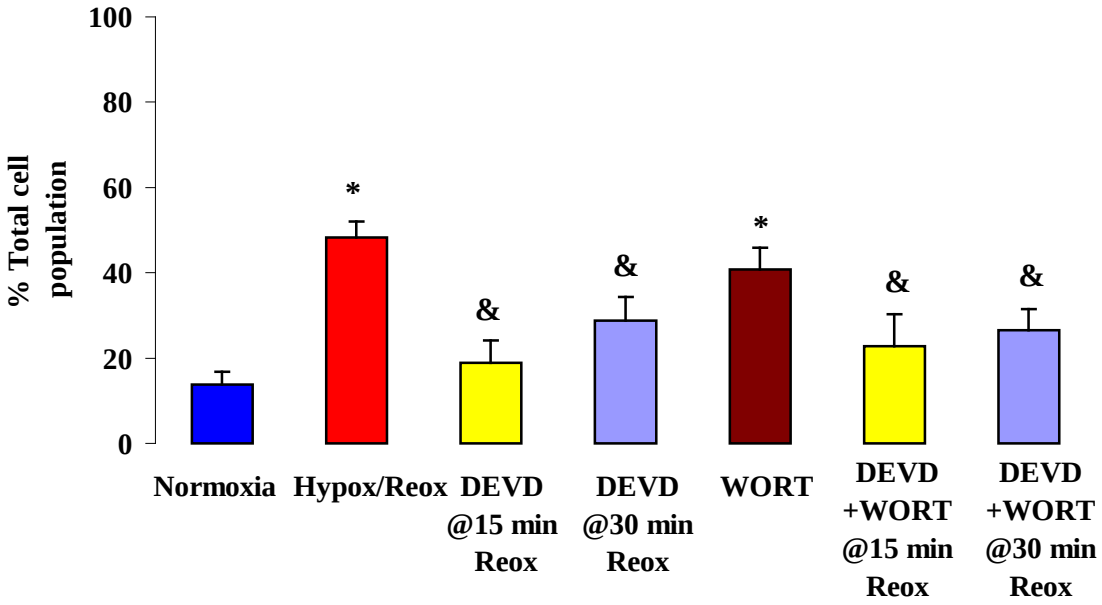


Figure 5.16: Assessment of myocyte apoptosis in isolated adult rat cardiomyocytes subjected to 6 hours hypoxia and 18 hours of reoxygenation. The specific caspase-3 inhibitor (DEVD, 25µM) was added at 15 minutes and 30 minutes after starting reoxygenation in the presence and absence of the of PI3-Akt inhibitor; Wortmannin (WORT, 100nM). Results are shown as mean + SEM and are expressed as a percentage of the total cells counted.* p<0.05 *vs*. Normoxia, & p<0.05 *vs*. Hypox/Reox Control, n=5.

5.3.2.2.5 The cytoprotective effect of the specific caspase-3 inhibitor (DEVD, 25µM) when added at 15 minutes and 30 minutes after starting repxygenation is not blocked by PI3-Akt inhibitor, Wortmannin (WORT, 100nM)

Mean % viable myyocte populations were significantly increased with DEVD (25 µM) treatment at 15 minutes and 30 minutes after starting reoxygenation in comparison to control Hypox / Reox myocytes (46.8 ± 3.3 %, 42.8 ± 6.5 % respectively *vs*. 18.2 ± 3.9 % Hypox/Reox control, Tukey's post-hoc test p<0.05) Figure 5.17. Treatment with PI3-Akt

inhibitor (WORT,100nM) simultaneously with DEVD at 15 minutes and 30 minutes after reoxygenation did not affect the protection afforded by DEVD, showing a significant increase in % viable myocytes compared to Hypox/Reox control myocytes (54.3 ± 9.7 %, 49.8 ± 6.2 % *vs* 18.2 ± 3.9 % Hypox/Reox control, Tukey's post-hoc test $p<0.05$) Figure 5.17.

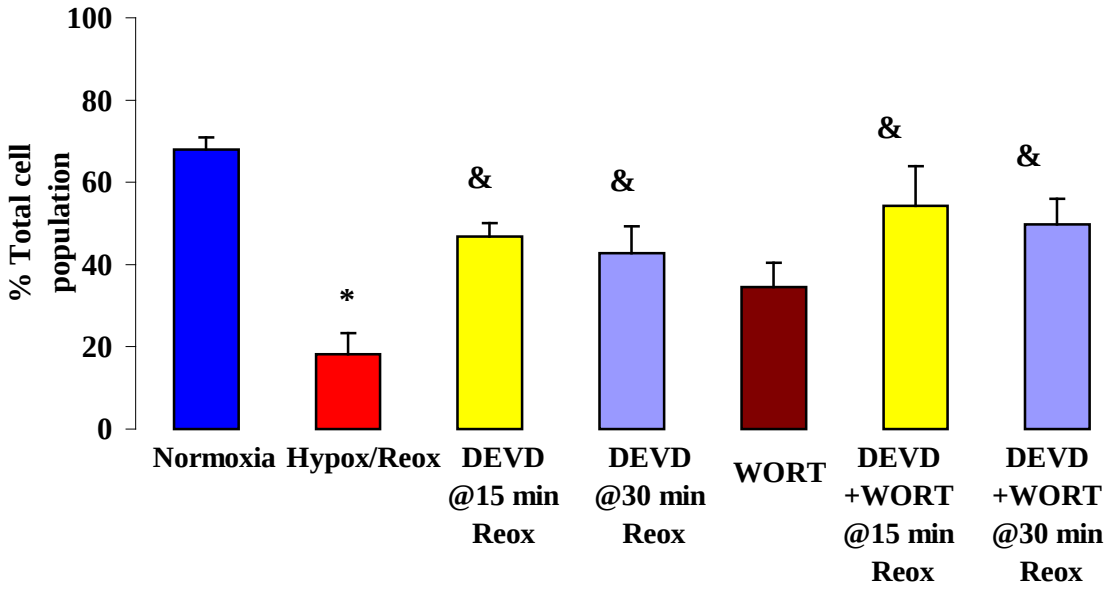


Figure 5.17: Assessment of myoctes viability in isolated adult rat cardiomyocytes subjected to 6 hours hypoxia and 18 hours of reoxygenation. The specific caspase-3 inhibitor (DEVD, 25µM) was added at 15 minutes and 30 minutes after starting reoxygenation in the presence and absence of the of PI3-Akt inhibitor; Wortmannin (WORT, 100nM). Results are shown as mean + SEM and are expressed as a percentage of the total cells counted.* $p<0.05$ *vs*. Normoxia, $ $p<0.05$ *vs*. Hypox/Reox Control, n=5.

5.3.2.2.6 The inhibitory effect of the specific caspase-3 inhibitor (DEVD, 25µM) when added at 15 minutes and 30 minutes after starting reoxygenation on caspase-3 activity level is not blocked by PI3-Akt inhibitor, Wortmannin (WORT, 100nM)

Results showed that there is a significant reduction in caspase-3 activity level after treatment with the specific caspase-3 inhibitor (DEVD, 25 µM) administered at 15 minutes and 30 minutes after starting reperfusion when compared to control myocytes exposed to

hypoxia and reoxgenation conditions (145.2 ± 54.4 %, 136.8 ± 22.8 % respectively *vs* 375.5 ± 90.9 % Hypox/Reox, Tukey's post-hoc test $p<0.05$) Figure 5.18. However, adding PI3-Akt specific inhibitor; Wortmannin (WORT, 100nM) simultaneously with DEVD at 15 minutes and 30 minutes after starting reoxygenation did not block DEVD mediated inhibitory effect on caspase-3 activity (190.7 ± 31.9 %, 131.3 ± 22.0 % rspectively *vs* 375.5 ± 90.9 % Hypox/Reox, Tukey's post-hoc test $p<0.05$) Figure 5.18.

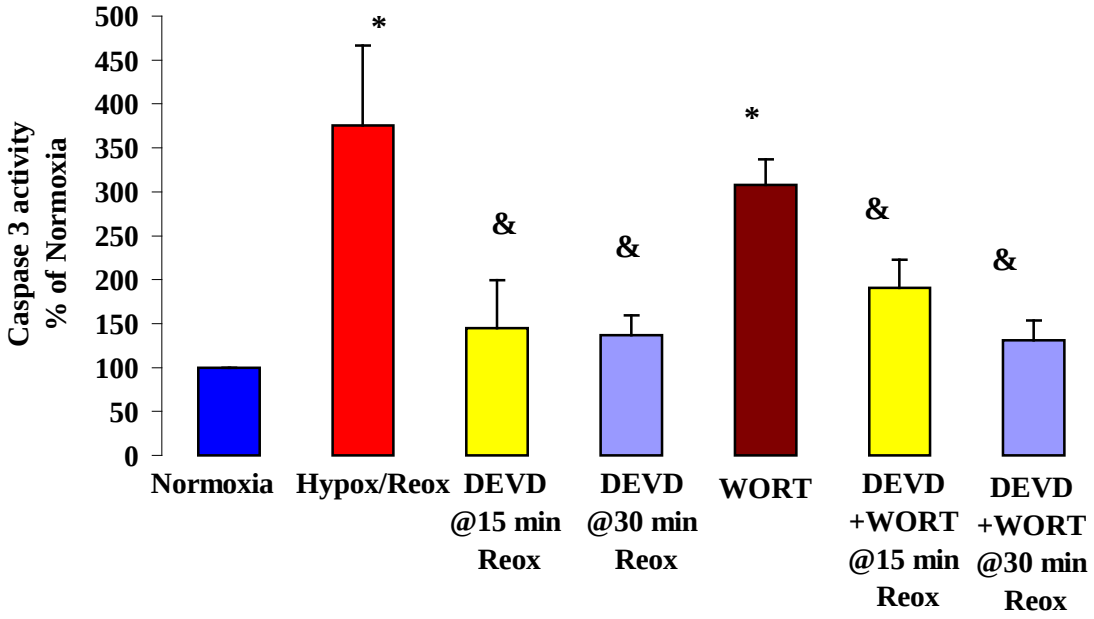


Figure 5.18: Caspase 3 activity in isolated adult rat cardiac myocytes subjected to 6 hours of hypoxia followed by 18 hours of reoxygenation. The specific caspase-3 inhibitor (DEVD, 25µM) was administered at 15 minutes and 30 minutes after starting reoxygenation in the presence and absence of the of PI3-Akt inhibitor; Wortmannin (WORT, 100nM). Results are shown as mean + SEM and are expressed as a percentage of the normoxia control values. * $p<0.05$ *vs*. Normoxia, & $p<0.05$ *vs*. Hypox/Reox Control, n=5.

5.3.2.2.7 Results summary

In summary both the broad spectrum caspase inhibitor (ZVAD) and the specific caspase 3 inhibitor (DEVD) resulted in significant cardioprotection when administered at 15 min and 30 min after starting reperfusion by siginicant reduction in infarction size within the risk area, significant reduction in apoptotic and siginificant reduction in intracellular caspase 3 acitivity. This cardioprotection was not mediated via PI3-Akt kinase pathway as co administration of PI3-Akt inhibitor (WORT, 100nM) did not block their cardioprotection at 15 min and 30 min as shown in table 5.2. The same control ischemia reperfusion group was used in all experimental studies.

Group	I/R %	Viability	Apoptosis	Caspase 3 activity (%of Normoxia)
Normoxia		68.0± 2.9	13.8± 3.0	100%
Control Ischemia/Rep	54.2±6.6	18.2±5.1	48.2± .8	375.5±90.8
ZVAD@15min Rep	30.5±3.4	42.1± 6.5	26.8±1.7	123.8±13.7
ZVAD@30min Rep	31.6±4.0	47.0±3.6	28.5±1.9	138.5±29.6
DEVD@15min Rep	15.7±3.6	46.8±3.3	18.9±5.2	145.2±54.4
DEVD@30min Rep	18.5±3.7	42.8±6.5	28.8±5.5	136.0±22.9
WORT	50.0±4.4	34.6±5.8	41.0±5.1	308.0 ±37.8
ZVAD+WORT @15min Rep	29.6±4.7*	47.0 4.3*	23.7±4.6*	123.8±13.7*
ZVAD+WORT @30min Rep	26.2±6.6*	48.0±2.2*	20.3±4.2*	138.5±29.6*
DEVD+WORT @15min Rep	18.0±4.3*	54.3±9.7*	22.8±7.4*	190.7±31.9*
DEVD+WORT @30min Rep	18.9±3.6*	49.8±6.2*	26.5±5.1*	131.3±22.0*

Table 5.2 Summary table showing the effect of the broad spectrum caspase inhibitor (ZVAD) and the specific caspase 3 inhibitor (DEVD) on I/R%, cellular viability, apoptosis and intracellular caspase 3 activity when admisitered at 15 min and 30 min after starting reperfusion in the presence and absence of the of PI3-Akt inhibitor; Wortmannin (WORT, 100nM). Results are shown as mean ± SEM * $p<0.05$ *vs*. control ischemia reperfusion.

5.3.2 Western blot analysis

5.3.2.1 Mitochondrial Markers

Cytochrome c is localized to the mitochondrial intermembrane space. It is released from the mitochondrial intermembrane space into the cytosole after induction of apoptosis. In this study cytosolic samples were isolated from mitochondrial samples and then were tested for mitochondrial markers to evaluate for any mitochondrial damage. Citrate synthase is a mitochondrial matrix enzyme, which can be considered as a mitochondrial marker. Citrate synthase enzyme did not differ between experimental groups ($p>0.05$), thus indicating that release of mitochondrial matrix proteins into the cytosolic fraction was similar for all experimental groups (Figure 5.19).

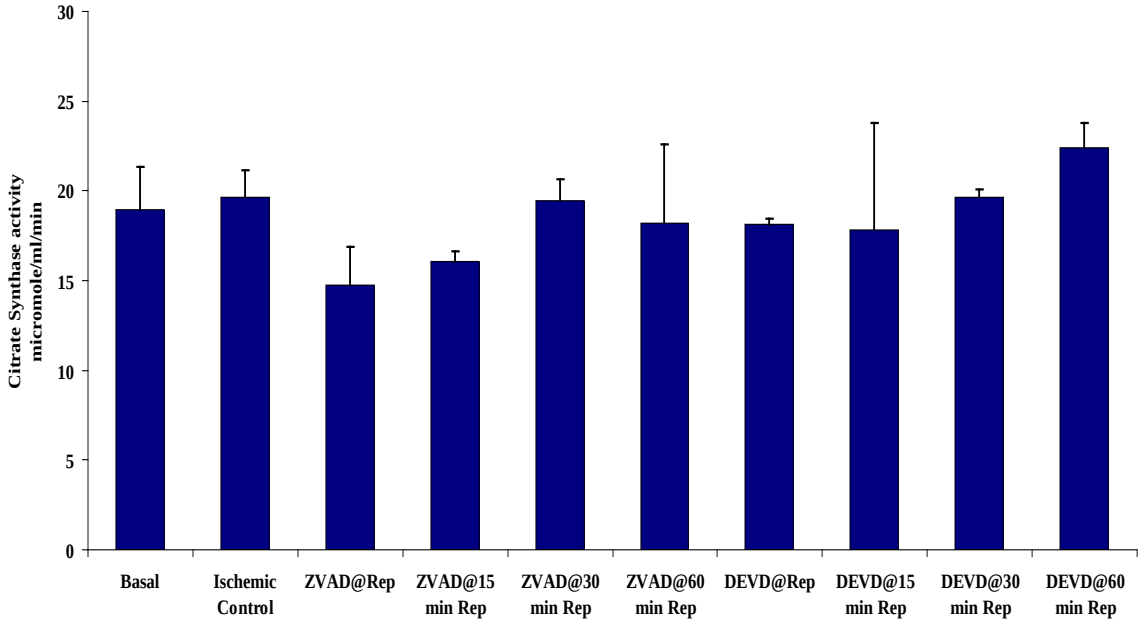


Figure 5:19 Assessment of citrate synthase activity level in isolated hearts subjected to 35 min ischemia followed by 120 minutes of reperfusion in control and either the broad spectrum caspase inhibitor (ZVAD, 0.1µM) or specific caspase-3 inhibitor (DEVD, 0.07µM) treated hearts at different time points during reperfusion throughout. Results are shown as mean + SEM of five individual experiments.

The voltage-dependent anion channel (VDAC) is an integral membrane protein present in the mitochondrial outer membrane, which is another mitochondrial marker. In this study we tested for VDAC using western blot analysis to assess any mitochondrial contamination of cytosolic samples. The VDAC was undetectable in all cytosolic samples when compared to mitochondrial samples (Figure 5.20).

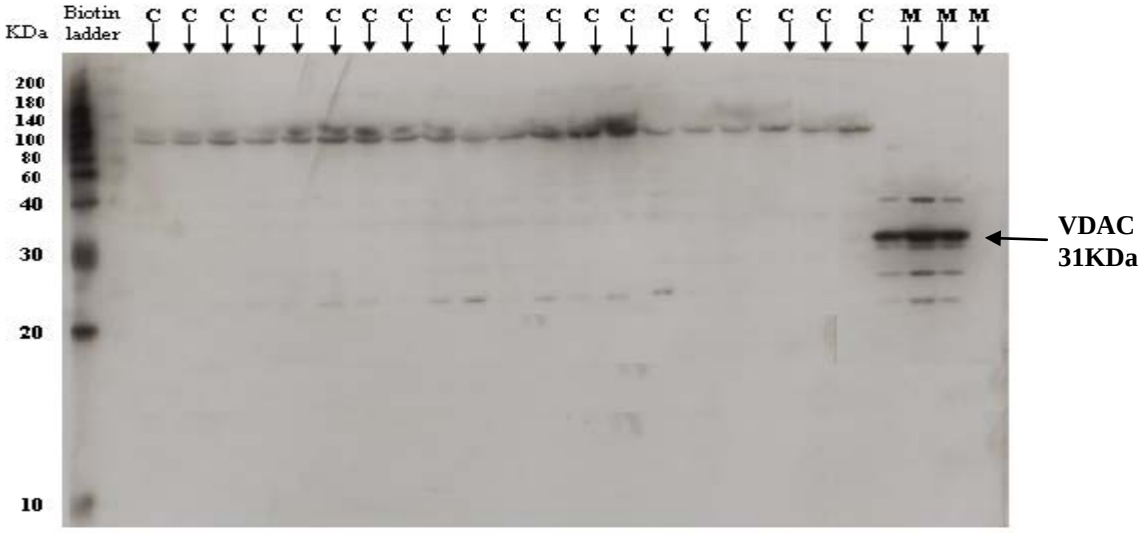


Figure5.20: Representative western blots for Voltage dependent anion channel (VDAC) in all cytosolic fractions of all experimental groups compared to mitochondrial fractions. Protein loaded was 30 µg per lane. C denotes cytosolic samples and M denotes mitochondrial samples.

5.3.2.2 Role of caspase inhibitors (ZVAD, 0.1µM or DEVD, 0.07µM) on cytochrome c release

In this study we are investigated wether caspase inhibitors mediated cardioprotection is dependent on mitochondrial cytochrome c pathway. Isolated rat hearts were exposed to 35 minutes ischemia followed by 120 minutes reperfusion, where caspase inhibitors (ZVAD, 0.1µM or DEVD, 0.07µM) added at different time points during reperfusion throughout. Tissue for western blot analysis for cytosolic cytochrome c collected at the end of 120 minutes reperfusion period. Cytosolic cytochrome c showed a significant increase in ischemic control hearts compared to basal non ischemic control hearts, which confirmed that cytochrome c is released from mitochondria to cytosole during ischemia reperfusion injury (75.0 ± 5.0 *vs* 38.0 ± 3.0 basal controls, LSD post hoc test $p<0.05$) Figure 5.21. However, neither caspase inhibitors significantly reduced cytochrome c release when added at any time points during reperfusion (LSD post-hoc test $p>0.05$) Figure 5.21.

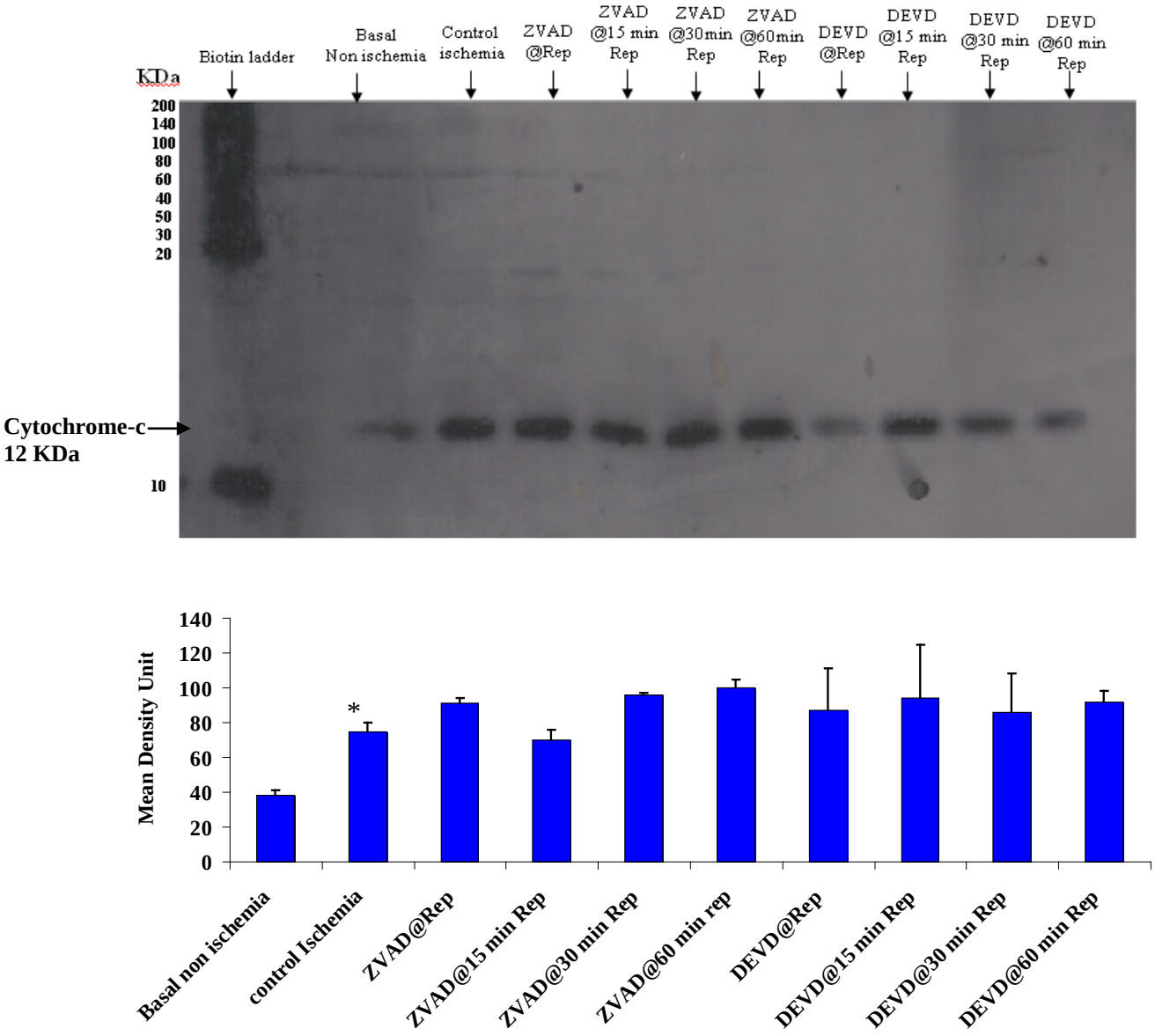


Figure 5.21: Assessment of cytosolic cytochrome c in isolated hearts subjected to 35 min ischemia followed by 120 minutes of reperfusion in control and either the broad spectrum caspase inhibitor (ZVAD, 0.1µM) or specific caspase-3 inhibitor (DEVD, 0.07µM) treated hearts. Results are shown as mean + SEM of fife individual experiments. *P<0.05 *vs*. basal non-ischemic control.

5.3.2.3 Phospho-Akt is upregulated with broad spectrum caspase inhibitor (ZVAD, 0.1µM) during early reperfusion.

In this study we aimed to invstigated wether caspase inhibitors mediated cardioprotection is mediated via PI3-Akt survival pathway. Hearts were harvested 5, 10, 20 and 120 minutes

after onset of reperfusion where the broad spectrum caspase inhibitor (ZVAD, 0.1μM) was administered at the onset of reperfusion in the presence and absence of PI3-Akt specific inhibitor (WORT, 100 nM). Western blot analysis was used to determine the protein expression levels of Phospho-Akt.

Hearts treated with ZVAD at reperfusion for 5 and 10 minutes demonstrated a significant increase in phosphorylated Akt in comparison to control ischemia reperfusion hearts at analogous time points (47.2 ±20.4, 64.3 ± 7.4 *vs*. 6.1 ± 3.9, 20.2 ± 1.9 ischemic controls at 5 and 10 minutes reperfusion, LSD post-hoc test $p<0.05$) Figure 5.22. However there was no significant difference in phospho-Akt expression level at 20 and 120 minutes in ZVAD treated hearts when compared to control ischemia reperfusion hearts at analogous time points (55.7 ± 12.0, 33.9 ± 16.4 *vs* 32.7 ± 28.7, 27.7 ± 14.5 ischemic controls at 20 and 120 minutes reperfusion, LSD post-hoc test $p>0.05$) Figure 5.22. Treatment with PI3-Akt specific inhibitor (WORT, 100nM) and ZVAD for 10 minutes of reperfusion showed a significant decrease in expression levels of phospho-Akt compared to ZVAD alone treated hearts at the same time point (4.5 ± 25.4 *vs*. 64.3 ± 7.4 ZVAD at 10 min reperfusion, LSD post-hoc test $p<0.05$) Figure 5.22. Treatment with WORT alone was comparable to same time Control hearts group (20.1 ± 6.0 *vs*. 20.2 ± 1.9 ischemic control at 10 min reperfusion, LSD post-hoc test $p>0.05$) Figure 5.22. The presence of additional non specific band in the western blot below the phosphor-Akt band shown in figure 5.22 was observed when home made gel used. Overall, a significant increase in phosphorylated Akt protein expression was observed in ZVAD treated hearts at 5 and 10 minutes time points compared to analogous controls and a decrease in expression was seen in combination treatment with WORT.

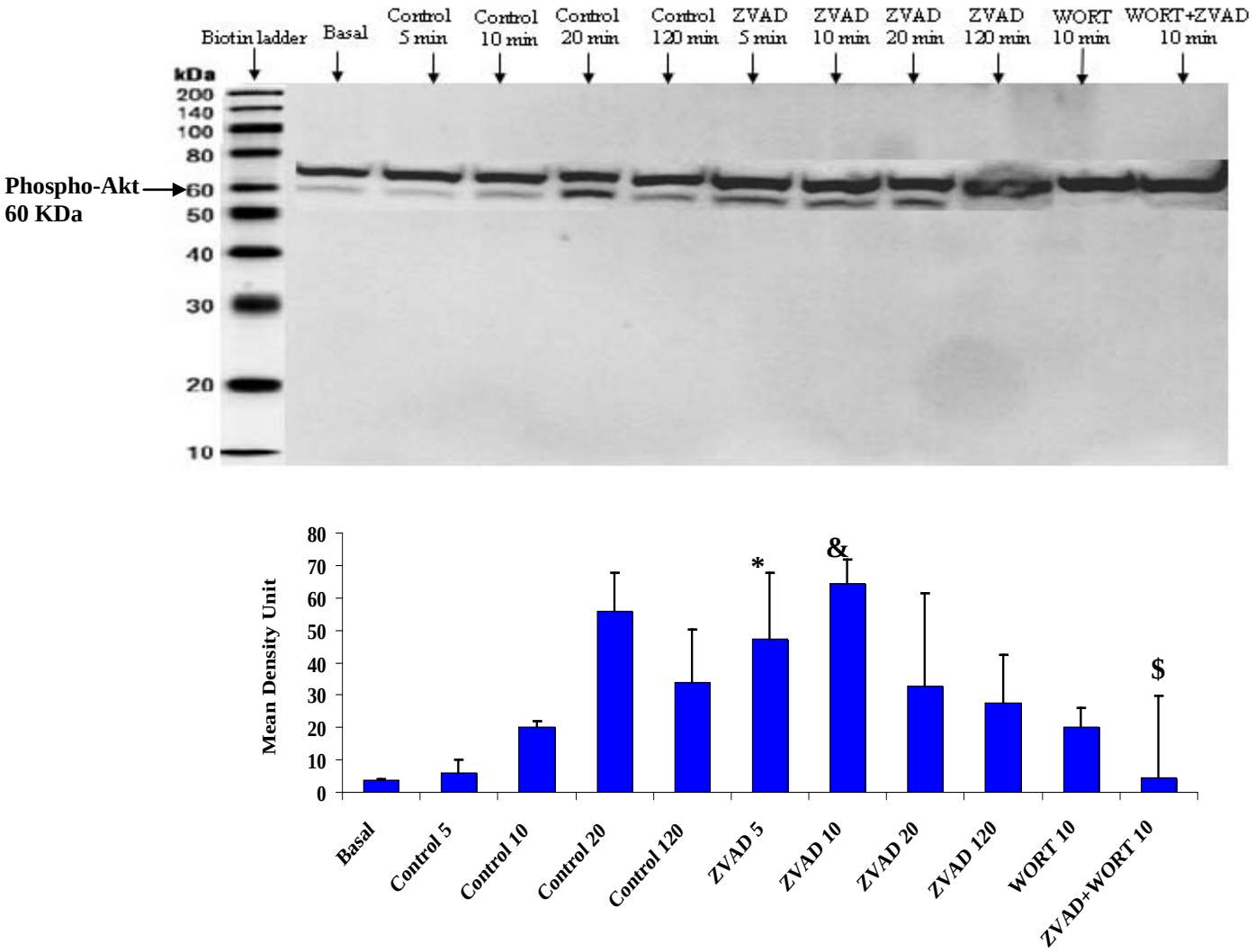


Figure 5.22: Assessment of phospho-Akt phosphorylation in isolated hearts subjected to 35 min ischemia followed by 5, 10, 20 or 120 minutes of reperfusion in control and the broad spectrum caspase inhibitor (ZVAD, 0.1µM) treated hearts. The ZVAD was administered at reperfusion in presence and absence of the PI3K inhibitor Wortmannin (WORT, 100nM). Results are shown as mean + SEM of three individual experiments. *P<0.05 *vs*. Control 5 min Reperfusion. & P<0.05 *vs*. Control 10 min Reperfusion. $ P<0.05 *vs*. ZVAD 10 min Reperfusion.

5.3.2.4 Phospho-Akt is upregulated with specific caspase-3 inhibitor (DEVD, 0.07µM) during early reperfusion

Administration of the specific caspase-3 inhibitor (DEVD, 0.07µM) at reperfusion significantly increases expression of phosphorylated-Akt after 5min (49.8 ± 20.2 *vs* 20.1 ±

6.2 ischemic control, LSD post-hoc test $p<0.05$, Figure 5.23) and 10 min (50.1 ± 11.7 *vs* 36.1 ± 15.4 ischemic control, LSD post-hoc test $p<0.05$, Figure 5.23) of reperfusion compared to non treated time matched ischemic controls. This up regulation of phospho-Akt by DEVD after 10 minutes of reperfusion was abrogated by the co-administration of the specific Akt inhibitor (WORT, 100nM) (50.1 ± 11.7 *vs* 19.7 ± 4.2, LSD post hoc test $p<0.01$, Figure 5.23). However, there was no significant difference in phospho-Akt expression level at 20 minutes reperfusion with DEVD when compared to control ischemia reperfusion hearts at analogous time point (35.4 ± 20.8 *vs* 64.4 ± 34.9 ischemic control at 20 minutes, LSD post-hoc test $p>0.05$, Figure 5.23). At 120 minutes reperfusion with DEVD, the expression level of phospho-Akt was significantly decreased when compared to ischemic control at matched time point (20.4 ± 5.3 *vs* 46.8 ± 8.2, LSD post-hoc test $p<0.05$, Figure 5.23). In this western blot the non specific band was not seen because this gel was purchased from Bio-Rad (Hemel Hempstead, UK) which could be attributed to more accurate resolution of the gel during protein transfer.

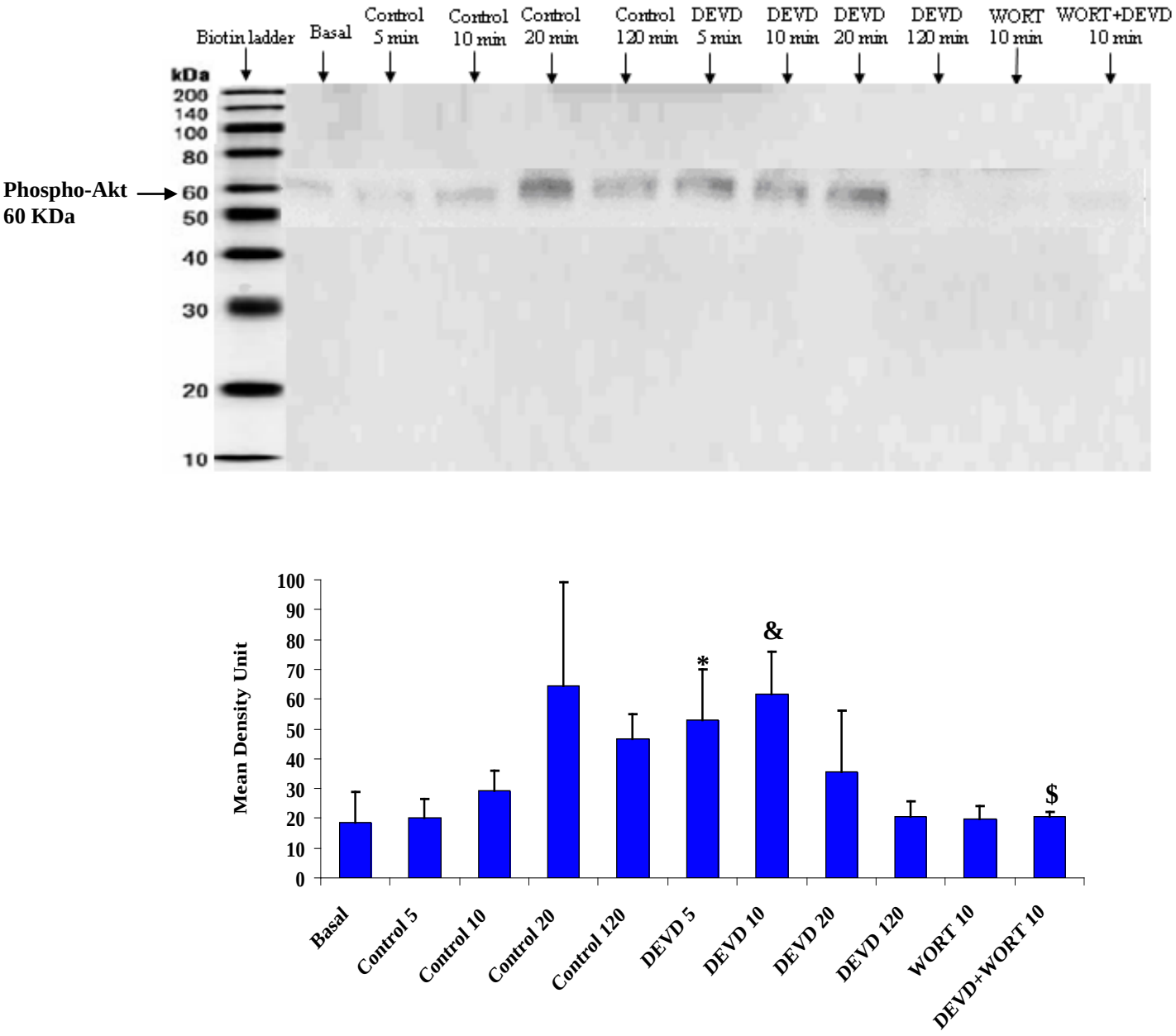


Figure 5.23: Assessment of phospho-Akt phosphorylation in isolated hearts subjected to 35 min ischemia followed by 5, 10, 20 or 120 minutes of reperfusion in control or the specific caspase-3 inhibitor (DEVD, 0.07µM) treated hearts. The DEVD was administered at reperfusion in presence and absence of the PI3K inhibitor Wortmannin (WORT, 100nM). Results are shown as mean + SEM of three individual experiments. *P<0.05 *vs.* Control 5 min Reperfusion. &P<0.05 *vs.* Control 10 min Reperfusion. .$ P<0.05 *vs.* DEVD 10 min Reperfusion.

5.4 Discussion

In this study we demonstrated that the broad spectrum caspase inhibitor (ZVAD) and the specific caspase-3 inhibitor (DEVD) were cardioprotective when administered immediately after starting reperfusion. Coadministration of wortmannin, inhibitor of PI3-Akt survival pathway abolished the cardioprotective effect of aforementioned caspase inhibitors by significant increase in infarction size, apoptosis, necrosis and caspase-3 activity level. These results can suggest that caspase inhibitors mediated cardioprotection could be hypothesized to be mediated via PI3-Akt survival pathway. However, cardioprotection offered by delayed administration of aforementioned caspase inhibitors, 15 minutes and 30 minutes after starting reperfusion was shown to be independent of PI3-Akt survival pathway.

5.4.1 Role of mitochondrial pathway on caspase inhibitors mediated cardioprotection

The mechanism by which apoptosis is induced by ischemia and/or reperfusion is still unclear. Caspase, which are considered as a key biochemical hallmark of apoptosis, can be activated by two main pathways (Budihardjo *et al*., 1999). One is mediated by cell surface death receptors, leading to autoactivation of caspase -8, which in turn activates downstream caspase such as caspase-3, 6 and 7 (Budihardjo *et al*., 1999). The second pathway is mediated by mitochondria, which release cytochrome c, which can associate with Apaf-1 (apoptosis protease activating factor) and pro-caspase-9, triggering the activation of downstream caspase-3 and apoptosis (Kromer *et al*., 1997). At present it is not clear which pathway is involved in initiation of apoptosis in myocardial ischemia/reperfusion injury. Several studies have implicated mitochondrial pathway in the cell injury in models of

myocardial infarction and cardimyocyte death (Vanden Hoek *et al*., 2003, Takamichi *et al*., 2004). In addition Date *et al*., (2003) showed that cardiomyocytes apoptosis predominantly proceeds *via* the mitochondrial pathway.

The results of our study confirmed the role of cytochrome c in ischemia reperfusion injury, as results showed significant increase of cytosolic cytochrome c in all groups exposed to ischemia and reperfusion compared to basal non-ischemic group. This suggests that mitochondrial pathway is involved in mediating apoptosis during myocardial ischemia reperfusion injury through cytochrome c release which is in agreement with previous literature. It is possible that this cytochrome c loss from mitochondrial matrix to cytosolic fraction could be an artifact due to mitochondrial damage during isolation procedure. However, this is unlikely as we found no significant change in citrate synthase activity (a mitochondrial matrix enzyme) between different experimental groups. Furthermore, VDAC (mitochondrial inner membrane protein) was undetectable in all cytosolic samples. In addition, inclusion of cyclosporine in the isolation buffer reduced the possibility of opening of mitochondrial transition pre and during the isolation procedure.

Previous studies showed that ischemia/reperfusion of the heart induces both caspase activation and cytochrome c release (Bialik *et al* 1999, De Moissac *et al* 2000, Borutaite *et al* 2001), however it is unclear whether cytochrome c release causes caspase activation or caspase activation causes cytochrome c release. Our study results showed that caspase inhibitors failed to inhibit cytochrome c release from mitochondria, which suggests that cytochrome c release from mitochondria is caspase independent. These results are in

agreement with previous studies that concluded that cytochrome c release is upstream of caspase activation (Bialik *et al*., 1999, de Moissac *at al*., 1999, Suzuki *et al*., 2001). Qin *et al* (2004) showed that caspase 8, 9 and 3 inhibitors failed to block cytochrome c release which is in agreement with the present study results. However they showed that caspase 2 inhibitor significantly blocked cytochrome c in chick cardiomyocytes exposed to simulated ischemia and reperfusion conditions and suggested a critical role of caspase 2 in initiating apoptosis in their model. In our present study we included a broad spectrum caspase inhibitor ZVAD, which is expected to have inhibitory effect on caspase 2 also; however we showed no significant effect on cytochrome c release. This discrepancy could be attributed to differences in cell type used as we used whole heart prepeartion compared to cheick cardiomyocytes grown in culture media used by Qin group. Also this discrepancy could be attributed to different synthetic caspase inhibitors used which vary in their specificity between manufactures, therefore we used two types of caspase inhibitors a broad spectrum one and more specific down stream caspase 3 inhibitos to compare their effect..

The possibility that cytochrome c release occurred during ischemic period should be considered. Therefore caspase inhibitors in our study model introduced at the onset of reperfusion period couldn't affect cytosolic cytochrome c level which has already being released during ischemic period. However this possibility could be ruled out as different studies reported that cytochrome c released only during reperfusion period using isolated heart model (Scarabelliand *et al*., 2002) and *in vivo* rat model (Lundberg & Szweda, 2004).

Lundberg & Szweda (2004) showed that a significant increase in cytosolic cytochrome c started at 30 minutes after starting reperfusion with greater overall increase between 30 and 60 minutes reperfusion with no further increase evident with extended reperfusion (120 minutes). Thus, the results of their experiments demonstrated a time-dependent increase of cytosolic cytochrome c with reperfusion. As we introduced caspase inhibitors at different time points (15 minutes, 30 minutes and 60 minutes) during reperfusion and found that there was no significant effect on cytochrome c release, we can rule out the effect of time distribution of cytochrome c release from mitochondria during reperfusion which again support that cytochrome c release from mitochondria is caspase independent. Therefore, the mechanism of caspase inhibitors cardioprotection might involve different route other than cytochrome c release from mitochondria.

5.4.2 Role of PI3-Akt cell survival pathway on caspase inhibitor mediated cardioprotecton during early phase of reperfusion

PI3-Akt and Erk1/2 cell survival pathways have been shown to be activated during ischemia reperfusion injury (Wu *et al*., 2000) and are considered as exciting innate cellular anti-apoptotic pathways of survival during myocardial ischemia reperfusion injury (Hausenloy & Yellon, 2004). Therefore, activation of these survival pathways may afford an opportunity for protecting the heart against ischemia reperfusion injury. A number of growth factors and pharmacological agents, together with ischemic postconditioing introduced immediately after starting reperfusion have been shown to induce cardioprotection via PI3-Akt and Erk1/2 survival pathway. These findings led Hausenloy & Yellon (2004) to postulate that these pathways might constitute a common pathway for

cardioprotection and termed them as reperfusion injury salvage kinase (RISK) pathway. Therefore caspase inhibitor mediated cardioprotection when administered during early reperfusion phase could also be via the RISK pathway. The results of the present study showed that there was an increase in phosph-Akt during the first 5 and 10 minutes of reperfusion with caspase inhibitors which further supports Hausenloy & Yellon theory. Using wortmannin, a potent irreversible inhibitor of PI3-Akt (Powise *et al*., 1994), further supports our results. Co-administration of wortmannin with both caspase inhibitors (ZVAD+DEVD) significantly abolished caspase inhibitors mediated cardioprotection via significant decrease in phosphorylation of Akt.

The present study showed that blocking Akt with wortmannin resulted in a significant increase in caspase 3 activity level in the caspase inhibitors treated groups, which suggest that Akt has an inhibitory effect on caspase-3 activity. This inhibitory effect could be through direct effect on caspase 3 at the cytosolic level or through Akt established effect on mitochondrial pathway. The effect of Akt on mitochondrial pathway of caspase activation is well estabilished through phosphorylation of Bad and Bax (pro-apoptotic proteins) and mitochondrial permeability transition pore (Hausenloy & Yellon, 2004). However, in this study, the Akt role in caspase inhibitors mediated cardioprotection is not through mitochondrial pathway as we mentioned earlier as there was no significant effect on mitochondrial cytochrome c release, therefore it is most likely via direct effect at the cytosolic level. These findings are in agreement with Wu *et al* (2000) as they showed that activation of PI3-Akt pathway led to suppression of caspase 3 activity independent of Bad phosphorylation. Uchiyama *et al* (2004) showed that Akt overexpression resulted in

inhibition of hypoxia reperfusion induced caspase 3 and 9 activation. Furthermore, PI3-Akt pathway has been found to inhibit caspase mediated apoptosis through direct phosphorylation of caspase 9 (Cardone *et al*., 1998).

Widmann *et al* (1998) demonstrated that caspase turn off survival signals in addition to activating death signals. They found for the first time that Akt and other signalling proteins were degraded during apoptosis by active caspase and that Akt degradation was inhibited by a caspase inhibitor using Jurkat and U937 cell model. Loss of these signalling proteins contributes significantly to caspase-mediated apoptosis. This study finding supports our study results, as we found that caspase inhibitors significantly increased the expression of Akt, which could be via elimination of the degradation effect of active caspase on Akt.

Wortmannin used in high dose of 100nM found to have inhibitory effect on other MAPKs signaling pathways (Davies *et al*., 2000). Therfore involvement of other signaling pathways in caspase inhibitors mediated cardipprotection could not be ruled out. Using lower doses of wortmanning like 5nM partilly blocked Akt activity (Khan *et al*., 2006) , that's why we decided to use 100nM in our studies baring in mind that it might have inhibitory effect on other pathways.

5.4.3 Cardioprotection by delayed administration of caspase inhibitors 15 minutes and 30 minutes after starting reperfusion is independent on PI3-Akt survival pathway

Cardioprotection by delayed administration of caspase inhibitors (ZVAD and DEVD) at 15 minutes and 30 minutes after starting reperfusion was not abolished by co-administration of

PI3-Akt specific inhibitor; Wortmannin. This suggests that the caspase inhibitors mediated cardioprotection during late phase of reperfusion is independent of PI3-Akt cell survival pathway. These findings are again consistent with the observation of Hausenloy & Yellon (2004) that PI3-Akt pathway played an important and critical role in cardioprotection during only the first minutes of reperfusion. However, Solenkova *et al* (2006) found that activation of PI3-Akt pathway is needed for the first 60 minutes of reperfusion for ischemic preconditioning (IPC) cardioprotective effect to take place, however, after 6o minutes reperfusion blocking the PI3-Akt pathway did not abolish the cardioprotecive effect of IPC using isolated rabbit heart model. They suggested that the hearts after 60 minutes don't need the support of PI3-Akt survival pathway. In our model of this study, we found that blocking the PI3-Akt survival pathway as early as 15 minutes did not abolish the caspase inhibitor cardioprotection. This discrepancy in the time window could be attributed to different cardioprotective strategies used. Precondiotioning cardioprotective strategy might have different mediators, growth factors and other signaling pathways affecting Akt activation compared to caspase inhibitors.

The involvement of another survival pathway, at delayed phase of reperfusion could be suggested. The Erk1/2 pathway is the other member in the RISK survival pathways. Erk1/2 activation has been demonstrated in the setting of ischemia reperfusion injury and can mediate cellular protection (Shimizu *et al*., 1998, Yue *et al*., 2000). Erhardt *et al*., (1999) showed that the B-Raf/MEK/Erk pathway interferes with apoptosis at the level of cytosolic caspase activation, downstream of the release of cytochrome *c* from mitochondria. Such postmitochondrial effect of Erk pathway at the level of caspase activation is consistent with

our study results, as we showed that caspase inhibitor cardiopotection is downstream of mitochondrial level. These findings raise the possibility that Erk signalling may play a significant role in mediating caspase inhibitors cardioprotection. Recently Hausenloy et al (2004) demonstrated that PI3-Akt and Erk1/2 pathways appear to interact in such a way that inhibiting one kinase cascade upregulates the activity of the other pathway, thereby acting as a compensatory safeguard, ensuring the signal for cellular protection is executed. Therefore the possibility of involvement of Erk1/2 pathway in mediating caspase inhibitors cardioprotection after inhibiting PI3-Akt pathway can not be excluded and needs further study.

5.4.4 Clinical implications and conclusion

It is important to establish the underlying mechanism of action of any drug and considered important in clinical field. This enables clinicians to decide about drug interaction and allows them to differentiate between drugs with synergistic or contradictory action. The recruitment of the PI3-Akt pathway at the time of reperfusion exerts powerful protection against ischemic–reperfusion injury, through various cellular protective mechanisms, including the inhibition of caspase, known executioners of apoptosis. Therefore, in a clinical setting, administrating Caspase inhibitors during early reperfusion provides a novel target for protecting the heart against lethal reperfusion injury through activation of PI3-Akt pathway, thereby delivering the powerful protective benefits associated with IPC and postconditioning.

CHAPTER 6

Broad spectrum caspase inhibitor improves post ischemic contractile function of isolated rat heart papillary muscle when administered during reperfusion and analysed using work loop technique

6.1 Introduction and objectives

One of the primary determinants of mortality following myocardial infarction is the extent of residual contractile function (Pasternak *et al*., 1988). Cellular death through apoptosis during ischemia reperfusion injury is thought to play an important role in the deterioration of myocardial contractile function, ventricular remodelling and heart failure after a myocardial ischemia reperfusion injury (Beltrami *et al*., 1994, Narula *et al*., 1996). Caspase play a crucial role in myocardial apoptosis (Bialik *et al*., 1997). Furthermore, caspase can also influence the contractile machinery of myocytes through cleavage of troponin and α-actinin, both are myofibrillar proteins (Haider *et al*., 1999, Communal *et al*., 2002), which can result in contractile dysfunction. We and several previous studies have found that broad spectrum and specific caspase inhibitor are cardioprotective in terms of reducing infarction/ risk ratio and apoptotic myocyte death when administered at the start of reperfusion (Mocanu *et al*., 2000). The effect of caspase inhibitors on functional recovery of the myocardium post ischemia is still under investigation and showed controversial results. Ysrbrough *et al*., (2003) showed promising results using broad spectrum caspase inhibitor. They showed that caspase inhibitors administerd at the start of reperfusion reduced post myocardial infarction left ventricular remodelling using *in vivo* pig model. Kovacs *et al*.,

(2001) again showed that broad spectrum caspase inhibition administered at onset of reperfusion for just 10 minutes, imporoved significantly the recovery of post ischemic cardiac function (coronary flow, aortic flow and left ventricular developed pressure). However they also reported that the hearts infused with specific caspase inhibitors (caspase 3 and caspase 9) showed no significant improvement in post ischemic cardiac function using isolated rat langendorff working model. On the other hand Rutten *et al*., (2001) using the same model, showed that caspase 3 inhibition significantly improved myocardial functional recovery post ischemia. Again Balsam *et al*., (2005) using in vivo mouse model, showed that caspase 3 inhibiton administerd at the onset of ischemia prevented ventricular diltation and dysfunction significantly. These controversial results could be attributed to the end target measured to assess ventricular function and the time of caspase inhibitors administration. Therefore, in this present study, we will investigate the effect of both broad spectrum (ZVAD) and specific caspase 3 (DEVD) inhibitors when administered at the onset of reperfusion period throughout on the power out put of the rat ventricular papillary muscle.

Contractile function assessment is strongly dependent on the index used (Chiu *et al*., 1989). Maximum power output has been proposed by Ford (1991) to be the more reliable index to assess the effects of inotropic interventions. He proposed three advantages of this index: 1) it is determined by interpolation rather than by extrapolation, therefore it is subjected to less error than extrapolated values like V_{max}. 2) it is sensitive to changes in both the force generating ability and the shortening capability of the muscle, in other words it measures the ability of the muscle to do work, which is the muscle's principal function and 3) it can

be normalized to muscle mass, which allows reliable comparison with results of other experiments results that are also normalized to muscle mass.

Working cardiac muscles undergo cyclical length changes, in which force development and shortening occur simultaneously and resultant work is produced (Semafuko & Bowie, 1975). Using the work loop technique allows *in vitro* measurement of maximum power output during cyclical length changes which simulates physiological contractions, making it a more reliable measure of *in vitro* performance than isometric or force-velocity studies (Josephson, 1985, James *et al.*, 1996). Layland *et al.*, (1997) demonstrated that the power and work-frequency relationships derived using work loop techniques are sensitive to changes in force, velocity, activation and relaxation rates. Therefore, they proposed that the work loop technique may represent a useful and sensitive approach for assessing the effects of pharmacological interventions on cardiac contractility.

Therefore, the aim of this experiment is to investigate the effect of the broad spectrum (ZVAD) and specific caspase-3 inhibitor (DEVD) administered at the start of reperfusion on post ischemic recovery of contractile function using an isolated rat ventricular papillary muscle model. Contractile function was measured using the work loop technique to give a realistic estimate of *in vivo* contractile performance.

6.2 Methods

Work loop technique was used to measure the power output of isolated rat ventricular papillary muscle as described in chapter 3 (section 3.7)

6.3 Results

6.3.1 Isometric studies

Mean isometric stress was not significantly different between experimental groups (One way ANOVA, $p>0.05$, table 1). These stress results were similar to those previously found for rat papillary muscle (Layland *et al*., 1994, 1995). The mean power output in the first 20 minutes stabilization period was not significantly different between experimental groups (One way ANOVA, $p>0.05$, table 1), which are within the same range of results found by Layland *et al.*, (1994,1995).

Group	Isometric stress ($kN\ m^{-2}$)	Mean power output ($W\ kg^{-1}$)
Baseline non-ischemic group (n=6)	48.4 ± 18.3	14.8 ± 3.3
Ischemic control group (n=6)	78.8 ± 28.5	23.4 ± 10.4
ZVAD (0.1 µM) (n=6)	49.0 ± 8.9	16.3 ± 2.4
ZVAD (2.5µM) (n=6)	49.2 ± 23.0	14.0 ± 2.8
DEVD (2.5 µM) (n=6)	48.9 ± 4.9	18.3 ± 1.6

Table 6.1: Baseline values (mean ± SEM) of isometric stress and mean power output during stabilization period for the different experimental groups.

6.3.2 Baseline non-ischemic contractile function of papillary muscle

The power output was stable for 150 minutes, signifying non deterioration in the papillary muscle preparation over the time course of the experiment (Figure 6.1).

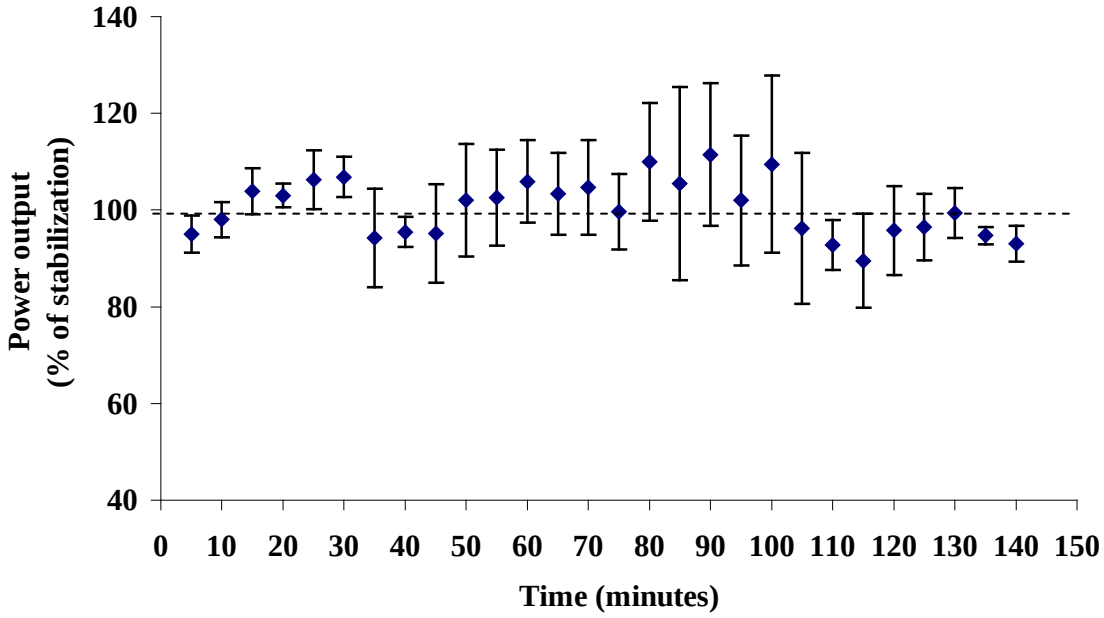


Figure 6.1: Power output of the rat heart papillary muscle exposed to normal oxygenated KHB for 150 minutes. Values are presented as mean percentages of the first 20 minutes stabilization period ± SEM. n=6

6.3.3 Effect of ischemia and reoxygenation on contractile function of papillary muscles (Ischemic control group)

Ischemia caused a significant rapid reduction in power output, reaching an 80% reduction within 15 minutes (Figure 2). Power output then gradually reduced to 9% of control stabilization power output (One-way ANOVA, Tukey's post hoc test $p<0.05$, Figure 2).

The power output recovered gradually during reoxygenation period to a maximum of 43% of control stabilization power output, which is significantly different to end ischemic values (One-way ANOVA, Turkey's post hoc test, $p<0.05$, Figure 6.2). However it didn't reach 100% recovery, this confirms the permanent damage caused by ischemia to the muscle, meaning the decline during ischemia was not only because of stunning effect.

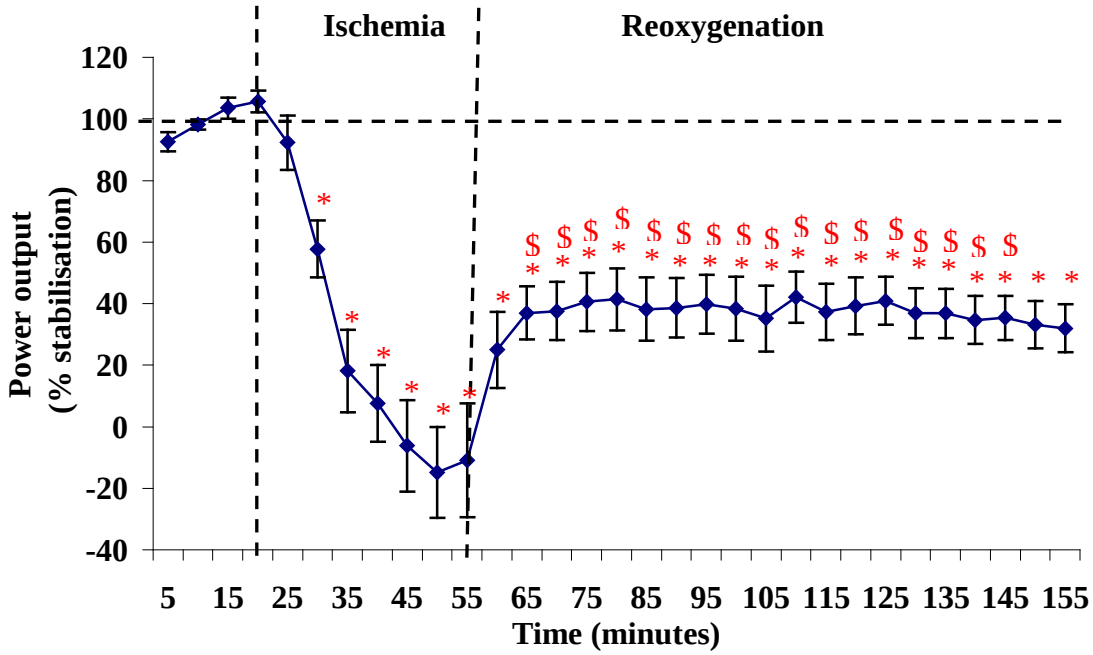


Figure 6.2: Changes in power output of the rat heart papillary muscle exposed to 35 minutes ischemia and 100 minutes reoxygenation. Values are presented as mean percentages of the stabilization period ± SEM. * $p<0.05$ vs. 20 min stabilization $ $p<0.05$ vs. 25 and 30 min ischemia, n=6.

6.3.4 The effect of the broad spectrum caspase inhibitor (ZVAD) on papillary muscle contractile function

The effect of the caspase inhibitor ZVAD was dose dependent in its ability to improve the contractile recovery post ischemia (Figure 6.3). The post ischemia contractile recovery of muscles treated with 0.10µM ZVAD was not significantly different when compared to ischemic control muscles (Two-way ANOVA, Tukey's post-hoc test, $p>0.05$, Figure 6.3),

however when the dose increased to 2.5µM, the contractile recovery post ischemia significantly improved (Two-way ANOVA, Tukey's post-hoc test $p<0.05$, Figure 6.3). The significant improvement started 20 minutes after starting reperfusion throughout reperfusion period when compared to matched ischemic control time points (One way ANOVA, Tukey's post-hoc test, $p<0.05$, Figure 6.3)

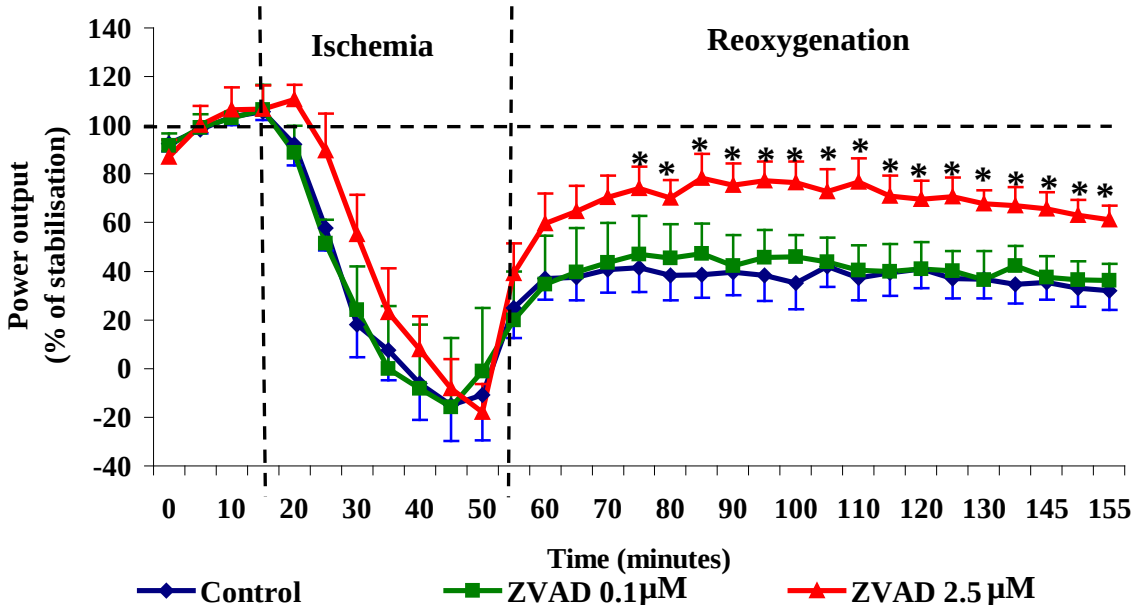


Figure 6.3: Changes in power output of the heart papillary muscles exposed to 35 minutes ischemia and 2 hours reoxygenation in the presence of different concentrations of broad spectrum caspase inhibitor; ZVAD (0.1µM, 2.5µM). Values are presented as mean percentages of the stabilization period ± SEM. * $p<0.05$ vs. ischemic control at matched time points, n=6.

6.3.5 The effect of the specific caspase-3 inhibitor (DEVD) on papillary muscle contractile function

The post ischemia contractile recovery of muscles treated with 2.5µM DEVD was not significantly different when compared to ischemic control muscles (Two-way ANOVA, Tukey's post-hoc test, $p>0.05$, Figure 6.4).

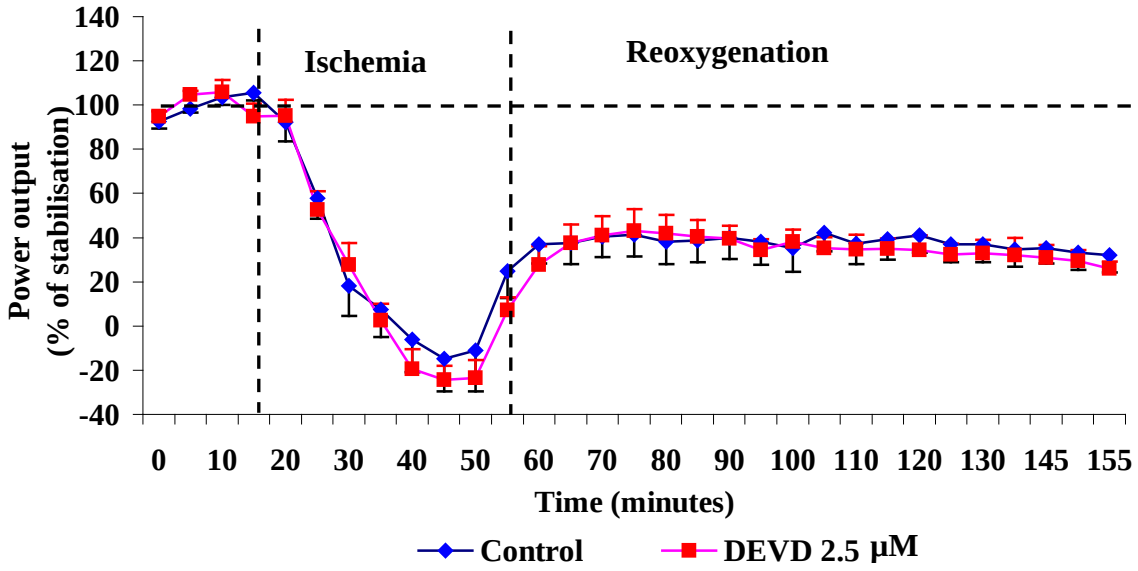


Figure 6.4: Changes in power output of the heart papillary muscles exposed to 35 minutes ischemia and 2 hours reoxygenation in the presence of specific caspase-3 inhibitor; DEVD (2.5µM). Values are presented as mean percentages of the stabilization period ± SEM, n=6.

6.4 Discussion

Cardiac contractile function is considered an important determinant of mortality following acute myocardial ischemia (Pasternak *et al*., 1988). Numerous studies have shown that apoptosis is a significant contributor to myocyte death during ischemia/reperfusion injury and have also highlighted the essential role of caspase as an executioner of apoptosis (Gottlieb *et al*., 1994, Saraste *et al*., 1997, Haung *et al*., 2000, Mocanu *et al*., 2000 and Zhao *et al*., 2000). In addition; these studies have also demonstrated that synthetic caspase inhibitors, both specific and broad spectrum, play a cardioprotective role by reducing infarction size and apoptotic myocyte death.

The main finding of the present study is that the broad spectrum caspase inhibitor (ZVAD) when administered at the start of reperfusion also plays another cardoprotective role by significantly improving the contractile recovery post ischemia in a dose dependent manner. However, the specific caspase-3 inhibitor (DEVD) at increased dose of 2.5μM failed to improve the contractile recovery post ischemia when administered at the start of reperfusion. These findings are in agreement with Kovacs et al., (2001) who showed that the non-specific caspase inhibition improved post-ischemic recovery, while selective caspase inhibition failed to improve post-ischemic cardiac function using langendorff working heart model and drugs administered at the start of reperfusion for just 10 minutes. However Rutten et al., (2001) showed that administration of caspase-3 inhibitor for 15 min during ischemia and 5 minute before starting reperfusionn improved contractile recovery using the same langendorff working heart model. This discrepancy in results could be

attributed to the different time of caspase inhibitor administration, local versus global ischemia and Rutten et al (2001) also used only 30 minutes reperfusion. However, our study is the first study to examine the direct effect of caspase inhibitors on cardiac muscle contractility using work loop technique (rather than using langendorff working heart model) and caspase inhibitors were added from the start of reperfusion, throughout reperfusion, which is clinically more feasible. In addition, our study model was the first to use the heart papillary muscle model. Definitive analysis of ventricular function requires more thorough understanding of the activation and contraction of each of its muscular elements like papillary muscles. Onset of contraction of the papillary muscles, as well as the epicardial muscle segments play more than a passive role in determining pressure development, valve action and flow patterns within the ventricle (Semafuko & Bowie, 1975).The ventricular papillary muscles contraction is generally assumed to be important for closure of atrioventricular valves. The isolated papillary muscle preparation has proven to be a useful tool for studying myocardial interaction and contraction (Wiegner *et al*., 1978, Shimizu *et al*., 1996d).

Apoptosis contributes significantly to cell death following myocardial ischemia reperfusion injury (Zhao *et al*., 2000, Gottlieb *et al*., 1994, Saraste *et al*., 1997), however the relationship between apoptosis, where caspase activation is the hallmark, and the deterioration of functional contractility of the heart following myocardial ischemia/reperfusion injury is not clear and has not been investigated thoroughly. Following myocardial infarction in humans, apoptosis has been found in the core of ischemic zone, border zone of the infarction and also in the viable myocardium away from

ischemic area (Saraste *et al*., 1997, Olivetti *et al*., 1996). Therefore, due to this wide spread of apoptosis, determining its exact contribution to functional deterioration post infarction is considered an important question to be answered. This could be answered indirectly by evaluating the effect of apoptosis inhibition on recovery of the functional parameters. We have previously (chapter 4) demonstrated increased rate of apoptosis with ischemia/reperfusion injury, which was significantly reduced by caspase inhibitors. Therefore, the effect of caspase inhibitor on contractile recovery can be attributed to their inhibitory effect on apoptosis. The broad spectrum caspase inhibitor at high dose of 2.5 µM afforded a significant improvement in the recovery of contractile function in reoxygenated rat ventricular papillary muscles. This improvement may have been due to reduction in the apoptotic myocytes as ZVAD has consistently been shown to significantly reduce apoptotic myocyte death following ischemia/reperfusion injury in different models (Yaoita *et al*., 1998, Holly *et al*., 1999, Mocanu *et al*., 2000). Therefore, the results of the present study indirectly confirm that apoptosis plays an essential role in the decline of functional contractility following myocardial ischemia and reoxyenation injury. However specific-caspase-3 inhibitor failed to show any significant improvement in post ischemic contractile function, despite its anti-apoptotic activity as shown in our previous work (chapter 4) and other studies (Okamura *et al*., 2000, Perrin *et al*., 2003). This may be due to the broad spectrum caspase inhibitor affected more caspase that might more specifically affect contractile machinery of the papillary muscle than did caspase-3 inhibitor in our study model. This is in agreement with Perrin *et al*., (2003) as they showed that specific caspase-3 inhibitor significantly inhibit apoptosis without any significant improvement in contractile function using langendorff perfusion model. However, Ruetten *et al*., (2001)

found that caspase 3 inhibition resulted in a functional contractile recovery independent of its apoptosis-inhibitory effects. This discrepancy between studies could be attributed to different factors including the functional model used, ischemic injury applied and time of drug administration. In addition, the caspase inhibitors dose was also different, as Ruetten *et al.*, (2001) used a low dose range of 0.1μM-1μM; this dose may be insufficient to reduce apoptosis to a statistically significant value as we and other studies shown previously (Gai *et al*., 2002).

In addition the possibility of inhibiting other proteases by broad spectrum caspase inhibitor such as calpain can not be ruled out. Different studies have shown calpain activation during ischemia-reperfusion injury (Yoshida *et al*., 1995a, Yoshida *et al*., 1995b), and its cleavage of the thin-filament regulatory protein troponin I with this being suggested as one of the mechanisms of myocardial stunning after ischemia reperfusion injury (Bolli *et al*., 1997).

The significant recovery of contractile function during treatment with the broad spectrum caspase inhibitor could be attributed, as mentioned above, to its ability to reduce the fraction of myocytes injured during hypoxia/reoxygenation injury as it has consistently been shown to significantly reduce apoptotic cell death following ischemia/reperfusion injury in different models (Yaoita *et al*., 1998, Holly *et al*., 1999), in addition it also reduces infarction size (Mocanu *et al*., 2000) which collectively may enhance contractile recovery. However, it could also be attributed to recovery of stunning effect post ischemia. Stunning defined as post-ischemic myocardial dysfunction that is fully reversible over time providing perfusion is not hindered (Bolli, 1990). In addition Bolli (1990) stated that in *in-*

vito experimental settings, the diagnosis of myocardial stunning should not be made unless reasonable assurance can be provided that the tissue in question is still entirely viable and that flow is normal or near-normal. Accordingly, our isolated muscle preparation is usually associated with significant cell death (TTC staining of papillary muscles showed significant infarction, data not shown), therefore stunning effect is unlikely. Furthermore, recovery in the present study was not complete and it reached a maximum of 59%, therefore, we can say that the recovery seen with broad spectrum caspase inhibitor is mainly due to cellular recovery rather than a transient stunning effect.

We observed a dose dependent effect of broad spectrum caspase inhibitor (ZVAD) on its ability to improve contractile recovery of papillary muscles post ischemia. ZVAD at 0.1µM concentration, has no significant recovery effect, however, with a relatively high dose of 2.5µM, significant improvement of contractile recovery was observed. The effective inhibitory concentration of ZVAD is variable among different studies. Cytoprotective effect was shown at a dose range from 10µM -100µM using isolated cardiomyocytes model (Gottlieb *et al*., 1996, Kang *et al*., 2000, Hai *et al*., 2001), on the other hand infarction size was significantly reduced at a dose as low as 0.1µM (Mocanu *et al.*, 2000). This variability could be attributed to the different models used and the end factor used to assess the effect of ZVAD. Specific caspase 3 inhibitor (DEVD) showed no significant effect at high dose of 2.5µM as compared to ZVAD. DEVD showed significant myocyte protection at a higher dose of 25µM as we demonstrated in chapter 4. Therefore, the possibility that DEVD can improve post ischemia functional recovery at high dose of 25µM is still there and needs further study.

In conclusion, developing pharmacological therapies administered adjunctively to standard reperfusion interventions to limit the severe consequences of ischemia reperfusion injury is still considered as a challenge. Our and previous results demonstrated that caspase inhibitors are cardioprotective by reducing myocyte death and also by improving post ischemic contractile recovery when administered at the start of reperfusion. This provides a promising route by which it is clinically feasible to treat patients presented with acute myocardial infarction.

CHAPTER 7

7.1 General Discussion

Limiting the impact of ischemic heart disease is of vital importance given the astronomical figures of ischemic heart related mortality and morbidity in the world. Early and complete reperfusion of the ischemic myocardium is essential for reduction of infarct size and preservation of cardiac function. This is currently achieved by thrombolysis, percutaneous transluminal coronary angioplasty (PTCA) and coronary artery bypass graft (CABG) surgery. The benefits of early reperfusion are well established but associated reperfusion injury is increasingly recognised to account for a proportion of the patient morbidity and mortality seen after reperfusion treatment. This reperfusion-induced injury can take the form of myocardial stunning, reperfusion arrhythmias, microvascular injury or lethal reperfusion injury. Lethal reperfusion injury is defined as "the death of myocytes, *alive at the time of reperfusion*, as a direct result of some aspect of reperfusion" (Hearse & Bolli 1992, Yellon & Baxter 2000). Therefore there is an imperative need for the development of pharmacological agents that can be used as adjunct therapies to limit ischemia reperfusion injury to be administered in a clinical setting.

Significant advances have been made in the past decade in understanding the underlying mechanisms of reperfusion injury and recognizing the distinct intracellular pathways involved in the mechanisms that influence cell death during reperfusion injury. Apoptotic cellular death found to contribute significantly to cell death during ischemia/reperfusion

injury. Our study results together with previous studies showed that apoptosis contribute significantly to myocytes death during ischemia reperfusion. Furthermore, our results showed that the contribution of apoptosis to myocytes death is significantly greater than necrotic myocyte death, which might suggest that apoptosis is the predominant mode of cell death during reperfusion injury. Therefore developing anti apoptotic therapies provides a novel target for protecting the heart against lethal reperfusion injury. Caspase represent the executionary machine of apoptosis (Cryns & Yaun, 1998) and responsible for the cleavage of the key cellular proteins that lead to the typical morphological changes observed in cells undergoing apoptosis, culminating in cellular death (Enari *et al*., 1996). Therefore caspase inhibitors could provide a promising therapeutic strategy to limit reperfusion injury.

Caspase inhibitors showed promising cardioprotective results in previous studies, however, their therapeutic window and whether their delayed administration after starting reperfusion will be cardioprotective, is still controversial. Our study results showed that broad and specific inhibition of caspase significantly attenuated myocardial infarction development in the risk zone, when administered immediately at the start of reperfusion, 15 min, 30 min and 60 min after starting reperfusion. These results were in agreement with Armstrong *et al* (2001) study results where they showed that caspase inhibitors (unspecified, still under investigation) significantly reduced infarct size even when administered one hour after the heart attack using *in vivo* rat model. Furthermore, our study results showed that caspase inhibitors introduced one hour after reperfusion are almost as cardioprotective as if they were introduced at the time reperfusion started. However, Li *et al* (2001) using isolated adult rabbit cardiomyocytes showed that caspase inhibitors (unspecified, labeled with

numbers) were partially protective if added 15 min after reperfusion and the cardioprotective effect was completely lost with a delay of 30 or 60 minutes. This discrepancy could be attributed to different types of inhibitors used and different models. In our study we tried to clarify this discrepancy by using both specific down stream caspase 3 inhibitor and a broad spectrum caspase inhibitor and also using different models, isolated heart langendorff model and isolated adult rat ventricular myocytes model.

Our study results demonstrated that the therapeutic window of myocardial infarction treatment with caspase inhibitors can be prolonged for at least one hour after starting reperfusion. This prolonged therapeutic window is clinically more feasible as patients are usually presented to hospital after the onset of ischemic attack and only at the start of reperfusion which is under clinician's control. Furthermore spontaneous reperfusion may occur in some patients before any hospital intervention due to dislodgment of the thrombus or relieved coronary spasm (Christian *et al.,* 1998) and therefore they presented to hospital at delayed time after starting spontaneous reperfusion. This therapeutic window is longer than for most cardioprotective drugs tested previously. A_3 agonist CL-IB-MECA lost its cardioprotective effect if administered 60 min after reperfusion (Hussain 2006) and A_{2a} agonist lost its cardioprotective effect when delayed 5 minutes only after starting reperfusions (Boucher *et al*., 2004). Administration of the insulin hormone 15 minutes after the onset of reperfusion resulted in a loss of the protection seen when administered at reperfusion (Jonassen *et al*., 2000).

Cardioprotection by delayed administration of caspase inhibitors mainly achieved through anti-apoptotic mechanism. These findings suggest that apoptosis contribution to cellular death during ischemia reperfusion injury is a continuous process and don't stop by starting reperfusion alone; however it might indicate that caspase activation could be initiated during ischemia but their irreversible cellular injury does not occur until later times. These suggestions can be supported by Vanden Hoek *et al*., (2003) results, where they reported that at least 2 hours of reperfusion was required to detect a significant level of caspase-3 activity required for fodrin cleavage to be detected. Furthermore human studies support our conclusion as they documented the occurrence of apoptosis in human heart specimens obtained from patients who died following myocardial infarction and they showed that apoptotic cells were significantly more numerous in the border zone of the infarcted tissue (Olivetti *et al.*, 1996, Saraste *et al.*, 1997) which suggests that apoptosis may be in part responsible for extending infarction over time after the onset of reperfusion (Olivetti *et al.*, 1996). Using animal model Zhao *et al*., (2001) found that myocardial apoptosis in the peri-necrotic myocardium progressively developed during the reperfusion period and was consistent with extension of infarction size and attenuation of contractile function and inhibition of apoptosis during reperfusion was associated with a reduction in infarction size and improvement in contractile function (Zhao *et al*., 2003). Therefore apoptosis can be considered as an important factor in exacerbation and extension of reperfusion injury and using caspase inhibitors could provide a potential therapeutic target.

Together with the significant reduction in infarction size and apoptotic cellular death, broad inhibition of caspase significantly improved the post ischemic contractile recovery of the

isolated adult rat papillary muscle when using the work-loop technique to measure the power output. These results were in agreement with previous studies were they showed that non-specific caspase inhibition improved left ventricular function and remodeling using in vivo rat and pig models (Yarbrough *et al*., 2003, Chandrashekhar *et al*., 2004). Even though haemodynamic parameters measured using langendorff model did not show any significant changes after caspase inhibitors administration. Using langendorff perfusion model to measure functional parameters is not truly reliable as usually they declined steadily during the experimental time course even without any ischemic intervention (Mocanu *et al*., 2000). However the working langendorff model allows measurement of cardiac output with different filling pressures and afterloads which provides more sensitive parameters of contractile function of the whole heart (Ytreuhs, 2000). In our laboratory experimental settings, it was difficult to use the working heart model due to lack of equipments and the expertise, therefore we decided to use the work loop technique to provide a more reliable assessment of caspase inhibitors effect on functional recovery using isolated ventricular papillary muscles. Using the work loop technique allows *in vitro* measurement of maximum power output during cyclical length changes of the muscle which simulates physiological contractions as working cardiac muscles undergo cyclical length changes, in which force development and shortening occur simultaneously and resultant work is produced, making it a more reliable measure of *in vitro* performance (Josephson, 1985, James *et al.,* 1996). In addition, our study model was the first to use the heart papillary muscle model. The ventricular papillary muscles contraction is generally assumed to be important for closure of atrio ventricular valves and the isolated papillary muscle

preparation has proven to be a useful tool for studying myocardial interaction and contraction (Wiegner *et al*., 1978, Shimizu *et al*., 1996d).

However specific inhibition of caspase 3 failed to improve the functional recovery of the isolated papillary muscle post ischemia. At present there is a little which could explain the discrepancies in the effect of the broad caspase inhibitor and the specific caspase inhibitor in their functional recovery effects. One explanation could be that the broad spectrum caspase inhibitor may have additional chemical characteristics which the specific caspase 3 inhibitor does not have. Broad caspase inhibitor could have inhibitory effect on caspase that might more specifically affect contractile machinery of the papillary muscle than did the specific one in our study model. In addition the possibility of inhibiting other proteases by the broad caspase inhibitor such as calpain can not be ruled out, as calpain has been shown to cleave the thin-filament regulatory protein troponin I, which has been suggested as one of the mechanisms of myocardial stunning after ischemia reperfusion injury (Bolli *et al*., 1997).

Despite the knowledge that caspase inhibitors can reduce injury in response to myocardial ischemia/reperfusion, very little is known about the intracellular signaling pathways that may be involved in mediating this protection. Caspases, can be activated by two main pathways, the extrinsic death receptor pathway and the intrinsic mitochondrial cytochrome c pathway. The role of the death receptor pathway in cardiac myocyte apoptosis is less well understood. Although Fas signalling has been implicated in cardiac hypertrophy (Badorff *et al*., 2002), heart failure (Levine *et al*., 1990) and calcium signaling (Felzen *et al*., 1998), its

role in cardiac myocyte apoptosis during ischemia reperfusion injury has been controversial. Some studies have demonstrated data suggesting that the CD95/Fas is directly involved in cell death after myocardial ischemia using isolated rat heart model and primary adult rat cardiomyocytes culture model (Jeremias *et al*., 2000) and *in vivo* model (Lee et al., 2003). However, Comiz *et al*., (2005) reported that mitochondrial permeability transition rather than activation of Fas plays a pivotal role in cardiomyocyte death after a prolonged ischemia-reperfusion insult in mice model. Moreover, mice treated systemically with an activating Fas antibody die from massive hepatocyte apoptosis but exhibit no cardiac pathology (Ogasawara *et al*., 1993). Scarabelli *et al*., (2004) (cited in Comiz *et al*., 2005) also demonstrated, by using the isolated rat heart model, that even after inhibition of caspase 8, release of cytochrome *c* and downstream activation of caspase 9 are seen during reperfusion, suggesting that mitochondrial damage and cardiomyocyte death is triggered independently of Fas activation. This suggests that cardiomyocytes apoptosis predominantly proceeds *via* the mitochondrial cytochrome c pathway (Date *et al*., 2003). The results of our study confirmed the role of mitochondrial cytochrome c pathway in ischemia reperfusion injury, as results showed significant increase of cytosolic cytochrome c in all groups exposed to ischemia and reperfusion compared to basal non-ischemic group which is in agreement with previous literature.

Broad and specific caspase inhibitors failed to inhibit cytochrome c release from mitochondria following ischemia reperfusion which is in agreement with previous studies (Bialik, 1999, de Moissac *at al*., 1999, Suzuki *et al*., 2001). This suggests that cytochrome c release from mitochondria is caspase independent and upstream of caspase activation step.

Therefore, caspase inhibitors mediated cardioprotection is not dependent on mitochondrial cytochrome c pathway and an alternative mechanism operating at the level of cytosolic caspase activation downstream of mitochondria can possibly be suggested.

Our study results showed that both broad and specific caspase inhibitors dependent cardioprotection during early phase of reperfusion is abrogated by the presence of the PI3-Akt inhibitor Wortmannin, implying PI3-Akt is required for caspase inhibitors dependent protection. Western blot studies also showed a significant increase in phoshorylation of PI3-Akt pathway during the first 5 and 10 minutes of reperfusion with both caspase inhibitors and using wortmannin significantly reduced PI3-Akt phosphorylation. Previous studies showed that activation of PI3-Akt survival kinase pathway constitutes a common survival pathway sufficient to induce a cardio-protective response during early reperfusion phase (Hausenloy & Yellon, 2004) and it seems from our results that caspase inhibitors shared the same cardioprotective signaling pathway. This conclusion could be supported by Wu *et al*., (2000) who showed a novel link between PI3-kinase pathway and suppression of caspase 3 activation independent of Bad phosphorylation using cardiac muscle cells. In addition Akt pathway also has been found to inhibit caspase mediated apoptosis through direct phosphorylation of caspase 9 (Cardone *et al*., 1998). Furthermore Widmann *et al* (1998) demonstrated that active caspase turn off survival signals by degradation of Akt and other signaling proteins using Jurkat and U937 cell model. Therefore caspase inhibitors cardioprotection during reperfusion could be suggested to be mediated through PI3-Akt pathway via elimination of the degradation effect of active caspase on Akt. (Figure 7.1). Utilization of wortmannin to dissect out the role of PI3-Akt poses limitations. Experimental

evidence showed that wortmannin inhibits PI 3-kinase at a concentration of 100nM, however wortmannin also inhibits other members of the MAPKs pathway at this concentration (Davies *et al*., 2000). Therfore the possibility of involvement of other cell signaling pathways could not be ruled out in our experimental study. On the other hand using wortmannin at lower doses partially blocked Akt (Khan *et al*., 2006), making it unfeasible to judge their effect. Therefore the kinetics of activation of Akt and the effective dose of wortmannin to attain significant Akt activation in the myocardium after ischemia reperfusion need to be addressed thoroughly and results should be taken with caution.

The cardioprotection by delayed administration of caspase inhibitors during reperfusion was not abolished by co-administration of PI3-Akt specific inhibitor; Wortmannin. This suggests that the caspase inhibitors mediated cardioprotection during late phase of reperfusion is independent of PI3-Akt cell survival pathway. The possibility that wortmannnin would have been degraded at later times of reperfusion because of its short half life of 10 minutes should be considered. However with our experimental setting of non-circulating continous infusion of the wortmainnin using langendorff perfusion system could rule out this possibility.The involvement of another survival pathway, at delayed phase of reperfusion could be suggested. Erk1/2 pathway was found to interfere with apoptosis at the level of cytosolic caspase activation, downstream of the release of cytochrome *c* from mitochondria (Erhardt *et al*., 1999). Furthermore Hausenloy et al (2004) demonstrated that PI3-Akt and Erk1/2 pathways appear to interact in such a way that inhibiting one kinase cascade upregulates the activity of the other pathway, thereby acting as a compensatory safeguard, ensuring the signal for cellular protection is executed.

Our study results and previous studies put caspase inhibitors in the front line of clinical research because of their promising cardioprotective results and prolonged therapeutic window, however several issues need to be clarified before applying them in clinical field. The long term effect of caspase inhibitors still not known, whether myocytes rescued by caspase inhibition continue to survive or may undergo early death because of injury already sustained. As we showed in our study and previous studies that caspase inhibitors failed to inhibit mitochondrial dysfunction and cytochrome c release which implies that the mitochondrial dysfunction occurred earlier than caspase activation. Furthermore some studies showed an increase in cellular necrosis associated with caspase inhibitors use (Suzuki *et al*., 2000). This might suggest that cellular fate is dependent on the extent of mitochondrial dysfunction more than extent of caspase activation. This area needs more investigation whether therapeutic strategies will be more effective if applied before or at the mitochondrial dysfunction stage or after caspase activation stage and whether combined interventions are more effective is also still not known and needs further study.In addition, Vercammen *et al*., (1998) showed evidence with noncardiac cell lines that caspase inhibitors accelerates endogenous generation of reactive oxygen species leading to cytotoxic action in association with caspase inhibition. All these studies highlighted an important issue regarding the long term effect of caspase inhibitors and their future use of caspase inhibitors in clinical field.

Capsaes activation and resultant apoptosis occurred in physiological process as well as pathological process, so, caspase inhibitors could potentially not only induce death in

unwanted pathological cells, but also might prevent cells damaged by lack of oxygen or other degenerative disease from killing themselves. Therefore using caspase inhibitors might result in unwanted side effects in clinical life. Better understanding of the entire caspase cascade that leads to apoptosis, along with the selection of specific targets and more selective inhibitors might improve the future clinical use of caspase inhibitors. Up-to-date there is no clinical studies used caspase inhibitors in ischemic heart disease treatment, however a phase I clinical trial of the pan-caspase inhibitor, IDN-6556 administered to patients with hepatic impairment showed the drug to be well tolerated and significantly reduced liver enzymes. Thus, the pan-caspase inhibitor, IDN-6556 is a potential agent for the treatment of human liver injury characterized by excessive apoptosis (Valentino *et al*., 2003). This study was based on animal studies that found that administration of IDN-6556 resulted in portal drug concentrations 3-fold higher than systemic concentrations, indicating a significant first-pass effect (Hoglen et al., 2004). Therefore before applying caspase inhibitors in treating ischemic heart disease further studies needed about the pharmacokinetics and pharmacodynamics and the doses required to get the significant drug level to affect the heart with minimum side effect.

Our study results can be considered important as one of the pre-clinical studies that tried to clarify the cardioprotective effect of caspase inhibitors and their mechanism of action before applying them in clinical studies for treatment of ischemic heart diseases. In short term basis caspase inhibitors proved to be cardioprotective by reducing infarct size, reducing apoptotic cellular death and improve post ischemic contrctile funtion. Our results

sugges that this cardioprotection is mediated through recruitment of the innate cell survival PI3-Akt signaling pathway.

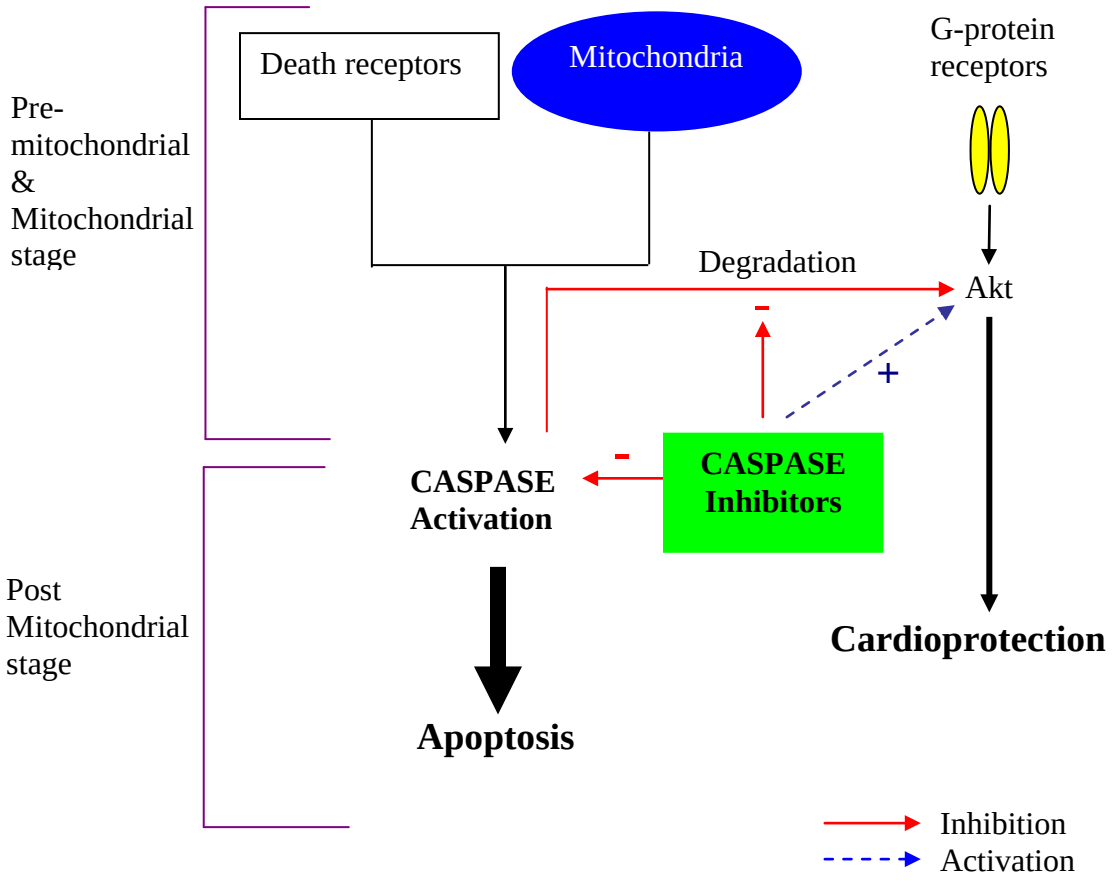


Figure 7.1: Proposed schematic pathway of caspase inhibitors mediated cardioprotection during ischemia reperfusion injury.

CHAPTER 8

8.1 Suggested examples of further investigations

- Caspase inhibitors showed cardioprotective results using different animal models; however their effect on human tissue/cells still limited and needs further investigation.This can be done using isolated human atrial trabecula preparation or human atrial muscle slices in tissue culture. The end points that can be measured are contractile function (force of contraction as a percentage of the baseline) in the isolated trabecula as an indirect measure of myocardial function. Assessment of cell death (by assay of creatine kinase release) and apoptosis (by annexin V labelling indicating an early event in apoptosis and using the modified TUNEL method with propidium iodide counterstaining indicating a later event in apoptosis). Different administration timing could be evaluted to evaluate caspase inhibitors theraputic window.

- Akt cell survival pathway has been shown to be involved in caspase inhibitors cardioprotection during early reperfusion, however its down stream mediators are not known yet and needs further investigation. Further studies needed to determine the role of P70S6K, BAD, NOS and mPTP. This can be done using specific inhibitors of these mediators in the presence and absence of caspase inhibitors. Western blot studies can be used to determine the protein expression level and whether they are affected by caspase inhibitors.

- Erk pathway is considered to be one of the cell survival signalling pathways. Different studies have shown that it is activated during ischemia reperfusion injury and involved in cardioprotection mediated by different cardioprotective strategies. However whether it could mediate caspase inhibitors cardioprotection still needs further investigation. This can be done by studying the effect of caspase inhibitors in the presence and absence of UO126 an Erk inhibitor. Western blot studies can be used to evalute phosphorylated Erk protein level.

- The long term effect of caspase inhibitors needs to be investigated before applying caspase inhibitors in the clinical field. Developing a reliable model to study the effect of caspase inhibitors over prolonged period of reperfusion considered important. *In vivo* model could provide a reliable model where caspase inhibitors effect can be studies over weeks and apoptosis assessment can be done using TUNEL technique.

- To verify whether therapeutic interventions applied at pre-mitochondrial and mitochondrial stage (befor caspase activation) is more effective than interventions applied at post mitochondrial stage (after caspase activation) needs further evaluation. Whether combined interventions are more effective is also still not known and needs further study. This can be done by studying the effect of caspase inhibitors in the presence and absence of mPTP inhibitor, cyclosporine.

References

Aikawa R, Nawano M, Gu Y, Katagiri H, Asano T, Zhu W, Nagai R, Komuro I (2000) 'Insulin prevents cardiomyocytes from oxidative stress-induced apoptosis through activation of PI3 Kinase/AKt.' *Circulation* 102, (23) 2873-2879.

Allender S, Peto V, Scarborough P, et al. 'Coronary heart disease statistics.' *British Heart Foundation: London 2006* [online] Available at http://www.heartstats.org/datapage.asp?id=5712 (accessed on 22 May 2006).

Alnemri E, Livingston D, Nicholson D, Salvesen G, Thornberry N, Wong W, Yuan J (1996) 'Human ICE/CED-3 protease nomenclature.' *Cell* 87, 171.

Altman R (2003) 'Risk factors in coronary atherosclerosis athero-inflammation: the meeting point.' *Thrombosis Journal* 4, 1-11.

Ansari B, Coates PJ, Greenstein BD, Hall PA (1993) 'In situ end-labelling detects DNA strand breaks in apoptosis and other physiological and pathological states.' *Journal of Pathology* 170, (1) 1-8.

Anversa P, Cheng W, Liu Y, Leri A, Redaelli G, Kajstura J (1998) 'Apoptosis and myocardial infarction.' *Basic Research in Cardiology* 93, (Suppl 3) 8-12.

Argaud L, Gateau-Roesch O, Raisky O, Loufouat J, Robert D, Ovize M (2005) 'Postconditioning inhibits mitochondrial permeability transition.' *Circulation* 111, 194-7.

Armstrong RC, Li F et al. (2001) **'**Caspase Inhibitors Reduce Infarct Size When Dosed Post-Reperfusion in a Rodent Cardiac Ischemia/Reperfusion Model.' *American Heart Association* 11/11/2001, 4:45 PM-5.00 PM.

Asano G, Takashi E, et al. (2003) 'Pathogenesis and protection of ischemia and reperfusion injury in myocardium.' *Journal of Nippon Medical School* 70, (5) 384-92.
Ashkenazi A, Dixit VM (1998) 'Death receptors: signalling and modulation.' *Science* 281, (5381) 1305-8.

Axford-Gatley R.A, Wilson GJ (1991) 'Reduction of experimental myocardial infarct size by oral administration of alpha tocopherol.' *Cardiovascular Research* 25, (2) 89-92.

Badorff, C, Ruetten H, Mueller S, Stahmer M, Gehring D, Jung F, Ihling C, Zeiher AM, Dimmeler S (2002) 'Fas receptor signaling inhibits glycogen synthase kinase 3 beta and induces cardiac hypertrophy following pressure overload.' *J Clin Invest* 109, 373-381.

Balakirev MW, Khramtsov VV, Zimmer G (1997) 'Modulation of the mitochondrial permeability transition by nitric oxide.' *European Journal of Biochemistry* 246, (3) 710-718.

Baxter GF, Ebrahim Z (2002) 'Role of bradykinin in preconditioning and protection of the ischaemic myocardium.' *British Journal of Pharmacology* 135, (4) 843-54.

Beltrami CA, Finato N, Rocco M, et al. (1994) 'Structural basis of end-stage failure in ischemic cardiomyopathy in humans.' *Circulation* 89, 151-163.

Berger AB, Sexton KB, Bogyo M (2006) 'Commonly used caspase inhibitors designed based on substrate specificity profiles lack selectivity.' *Cell Research* 16, (12) 961-3.

Bialik S, Geenen DL, Sisson IE, et al. (1997) 'The caspase family cysteine proteases mediate cardiac myocyte apoptosis during myocardial infarction.' *Circulation* 96, 1-552.

Bialik S, Cryns VL, Drincic A, Miyata S, Wollowick AL, Srinivasan A, Kitsis RN (1999) ‘The mitochondrial apoptotic pathway is activated by serum and glucose deprivation in cardiac myocytes.’ *Circulation Research* 85, 403-414.

Blomgren K, Zhu CL, Wang XY, Karlsson JO, Leverin AL, Bahr BA, Mallard C et al. (2001) ‘Synergistic activation of caspase-3 by m-calpain after neonatal hypoxia-ischemia.’ *Journal of Biological Chemistry* 276, 10191-10198.

Bogoyevitch MA, Gillespie-Brown J, Ketterman AJ, Fuller SJ, Ben-Levy R, Ashworth A, Marshall CJ, Sugden PH (1996) ‘Stimulation of the stress-activated mitogen-activated protein kinase subfamilies in perfused heart: p38/RK mitogen-activated protein kinases and c-Jun N-terminal kinases are activated by ischemia/reperfusion.’ *Circulation Research*, 79, 162-173.

Bolli R, Patel BS, Jeroudi MO, et al. (1988) ‘Demonstration of free radical generation in "stunned" myocardium of intact dogs with the use of the spin trap alpha-phenyl N-tert-butyl nitrone.’ *Journal of Clinical Investigation* 82, 476-85.

Bolli R, Jeroudi MO, Patel BS, et al. (1989) ‘Direct evidence that oxygen-derived free radicals contribute to postischemic myocardial dysfunction in the intact dog.’ *Proc Natl Acad Sci USA* 86, 4695–4699.

Bolli.R (1990) ‘Mechanism of myocardial “stunning”.’ *Circulation.* 82,723-738.

Bolli R, Marban E (1999) ‘Molecular and cellular mechanisms of myocardial stunning.’ *Physiol Rev* 79, 609-634.

Bonni A, Brunet A, West AE, Datta SR, Takasu MA, Greenberg ME (1999) ‘Cell survival promoted by the Ras-MAPK signaling pathway by transcription-dependent and -independent mechanisms.’ *Science* 286, (5443) 1358-1362.

Bopassa JC, Ferrera R, Gateau-Roesch O, Couture-Lepetit E, Ovize M (2005) 'PI 3-kinase regulates the mitochondrial transition pore in controlled reperfusion and postconditioning.' *Cardiovascular Research* 69, 178-185.

Borutaite A, Budriunaite A, Morkuniene R, Brown GC (2001) 'Release of mitochondrial cytochrome c and activation of cytosolic caspase induced by myocardial ischemia.' *Biochemica et Biophysica Acta* 1537, 101-109.

Borutaite A, Jekabsone RM, Brown GC (2003) 'Inhibition of mitochondrial permeability transition prevents mitochondrial dysfunction, cytochrome *c* release and apoptosis induced by heart ischemia.' *Journal of Molecular and Cellular Cardiology* 35, 357-366.

Brar BK, Jonassen AK, Stephanou A, Santilli G, Railson J, Knight RA, et al. (2000) 'Urocortin protects against ischemic and reperfusion injury via a MAPK-dependent pathway.' *Journal of Biological Chemistry* 275, (12) 8508-8514.

Briaud SA, Ding ZM, Michael LH, Entman ML, Daniel S, Ballantyne CM (2001) 'Leukocyte trafficking and myocardial reperfusion injury in ICAM-1/P-selectin-knockout mice.' *American Journal of Heart Circulation and Physiology* 280, (1) H60-H67.

Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, Hu JS, et al. (1999) 'Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor.' *Cell* 96, (6) 857-868.

Buja LM, Entman ML (1998) **'**Modes of myocardial cell injury and cell death in ischemic heart disease.' *Circulation* 98, 1355-1357.

Cardone HM, Roy N, Stennicke HR, Salvesen GS, Franke TF, Stanbridge E, et al. (1998) 'Regulation of cell death protease caspase-9 by phosphorylation.' *Science* 282, (5392) 1318-1321.

Cardone MH, Salvesen GS, Widmann C, Johnson G, Frisch SM (1997) 'The regulation of anoikis: MEKK-1 activation requires cleavage by caspase.' *Cell* 90, 315–323.

Cerretti DP, Kozlosky CJ, Mosley B, Nelson N, Van Ness K, Greenstreet TA, March C J, Kronheim SR, Druck T, Cannizzaro LA, et al. (1992) 'Molecular cloning of the interleukin-1β converting enzyme.' *Science* 256, 97-100.

Chandrashekhar Y, Sen S, Anway R, Shuros A, Anand I (2004) 'Long-term caspase inhibition ameliorates apoptosis, reduces myocardial troponin-I cleavage, protects left ventricular function, and attenuates remodeling in rats with myocardial infarction.' *J Am Coll Cardiol* 43, 295-301.

Chaudhary PM, Eby MT, Jasmin A, Hood L (1999) 'Activation of c-Jun N terminal kinase/stress-activated protein kinase by overexpression of caspase 8 and its homologs.' *Journal of Biological Chemistry* 274, 19211-19219.

Chen M, He H, Zhan S, Krajewski S, Reed JC, Gottlieb RA (2001) 'Bid is cleaved by calpain to an active fragment in vitro and during myocardial ischemia/reperfusion.' *Journal of Biological Chemistry* 276, 30724-30728.

Chinnaiyan, AM, O'Rourke K, Tewari M, Dixit VM (1995) 'FADD, a novel death domain-containing protein interacts with the death domain of Fas and initiates apoptosis.' *Cell* 81, 505-512.

Chiu YC, Walley KR, Ford LE (1989) 'Comparison of the effects of different inotropic interventions on force, velocity and power in rabbit myocardium.' *Circulation Res*. 65, 1161-1171.

Christian TF, Milavetz JJ, Miller TD, Clements JP, Holmes DR, Gibbons RJ (1998) 'Prevalence of Spontaneous Reperfusion and Associated Myocardial Salvage in Patients with Acute Myocardial Infarction.' *American Heart Journal* 135, (3) 421-427.

Cohen MV (1989) 'Free radicals in ischemic and reperfusion myocardial injury: is this the time for clinical trials?' *Annals of Internal Medicine* 111, 918-931.

Coles HSR, Burne JF, Raff MC (1993) 'Large-scale normal cell death in the developing rat kidney and its reduction by epidermal growth factor.' *Development* 118, 777-784.

Communal C, Sumandea M,, de Tombe P, Narula J, Solaro RJ, Hajjar RJ (2002) 'Functional consequences of caspase activation in cardiac myocytes.' *Proc Natl Acad Sci USA* 99, (9) 6252-6256.

Crompton M. (1999) 'The mitochondrial permeability transition pore and its role in cell death.' *Biochemistry journal* 341, 233-249.

Cross TG, Scheel-Toellner D, Henriquez NV, Deacon E, Salmon M, Lord JM (2000) 'Serine/threonine protein kinases and apoptosis.' *Experimental Cell Research* 256, (1) 34-41.

Cryns VL, Yuan J (1998) 'Proteases to die for.' *Genes &Development* 12, 1551-1570.

Cuevas P, Reimers D, Carceller F, Martinez-Coso V, Redondo-Horcajo M, Saenz DT, et al. (1997) 'Fibroblast growth factor-1 prevents myocardial apoptosis triggered by ischemia reperfusion injury.' *European Journal of Medical Research* 2, (11) 465-468.

Darling CE, Jiang R, Maynard M, Whittaker P, Vinten-Johansen J, Przyklenk K (2005) 'Postconditioning via stuttering reperfusion limits myocardial infarct size in rabbit hearts: role of ERK ½.' *American Journal of Physiology Heart and Circulatory Physiology* 289, H1618-H1626.

Date T, Mochizuki S, Belanger AJ, Yamakawa M, Luo Z, Vincent KA, Cheng SH, Gregory RJ, Jiang C (2003) 'Differential effects of membrane and soluble Fas ligand on cardiomyocytes: role in ischemia/reperfusion injury.' *Journal of Molecular and Cellular Cardiology* 35, 811-1821.

Datta K, Bellacosa A, Chan TO, Tsichlis PN (1996) 'Akt is a direct target of the phosphatidylinositol 3-kinase. Activation by growth factors, v-src and v-Ha-ras, in Sf9 and mammalian cells.' *Journal of Biological Chemistry* 271, (48) 30835-30839.

Davidson SM, Hauensloy D, Duchen MR, Yellon DM (2006) 'Signaling via the reperfusion injury signaling kinase (RISK) pathway links closure of the mitochondrial permeability transition pore to cardioprotection.' *International Journal of Biochemistry & Cell Biology* 38, (3) 414-9.

Davies SP, Reddy H, Caivano M, Cohen P (2000) 'Specificity and mechanism of action of some commonly used protein kinase inhibitors.' *Biochem. J.* 351, 95-105.

de Boer LW, Rude RA, Kloner JS, Ingwall PR, Maroko MA, Branwald E (1983) 'A flow- and time-dependent index of ischemic injury after experimental coronary occlusion and reperfusion.' *Proc Natl Acad Sci USA* 80, 5784-5788.

de Boer MJ, Ottervanger JP, van't Hof AW, Hoorntje JC, Suryapranata H, Zijlstra F (1994) 'Reperfusion therapy in elderly patients with acute myocardial infarction: a randomized comparison of primary angioplasty and thrombolytic therapy.' *American College of Cardiology* 39, (11) 1723-8.

Deutsch E, Berger M, Kussmaul WG, HirshfeldJW Jr, Herrmann HC, Laskey WK (1990) ‘Adaptation to ischemia during percutaneous transluminal coronary angioplasty. Clinical, hemodynamic and metabolic features.’ *Circulation* 82, 2044-2051.

Duchen MR (2000) **‘**Mitochondria and Ca2+ in cell physiology and pathophysiology.’ *Cell Calcium* 28, 339-48.

Dumont EA, Hofstra L, van Heerde WL, van Den ES, Doevendans PA, DeMuinck E, et al. (2000) ‘Cardiomyocyte death induced by myocardial ischemia and reperfusion: measurement with recombinant human Annexin-V in a mouse model.’ *Circulation* 102, 1564-1568.

Earnshaw WC (1995) ‘Apoptosis: lessons from in vitro systems.’ *Trends in Cell Biology* , 5, (6) 217-20.

Enari M, Sakahira H, Yokoyama H, Okawa K, Iwamatsu A, Nagata S (1998) ‘A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD.’ *Nature*, 391, (6662) 43-50.

Enari M, Talanian RV, Wong WW, Nagata S (1996) ‘Sequential activation of ICE-like and CPP32-like proteases during Fas-mediated apoptosis.’ *Nature* 380, (6576) 723-6.

Fan T, Han L, Cong R, Liang J (2005) ‘Caspase family proteases and apoptosis.’ *Acta Biochimica et Biophysica Sinica* 37, (11) 719-727.

Felzen, B, Shilkrut M, Less H, Sarapov I, Maor G, Coleman R, Robinson RB, Berke G, Binah O (1998) ‘Fas (CD95/Apo-1)-mediated damage to ventricular myocytes induced by cytotoxic T lymphocytes from perforin-deficient mice: a major role for inositol 1,4,5-trisphosphate.’ *Circ Res* 82, 438-450.

Flaherty JT, Pitt B, Gruber JW, et al. (1994) ‘Recombinant human superoxide dismutase (h-SOD) fails to improve recovery of ventricular function in patients undergoing coronary angioplasty for acute myocardial infarction.’ *Circulation* 89, 1982-1991.

Fliss H, Gattinger D (1996) ‘Apoptosis in ischemic and reperfused rat myocardium.’ *Circulation Research* 79, (5) 949-56.

Ford LE (1991) ‘Mechanical manifestations of activation in cardiac muscle.’ *Circulation Res.* 68, 621-637.

Forde RC, Fitzgerald DJ (1997) ‘Reactive oxygen species and platelet activation in reperfusion injury.’ *Circulation* 95, (4) 787-9.

Forman MB, Virmani R, Puett DW (1990) ‘Mechanisms and therapy of myocardial reperfusion injury.’ *Circulation* 81, IV69-IV78.

Frangogiannis N G, Youker, K A, Rossen R D, Gwechenberger M, Lindsey MH., Mendoza LH., Michael L H, Ballantyne CM, Smith C W, Entman ML (1998) ‘Cytokines and the microcirculation in ischemia and reperfusion.’ *Journal of Molecular and Cellular Cardiology* 30, 2567- 2576.

Frasch SC, Nick JA, Fadok VA, Bratton DL, Worthen GS, Henson PM (1998) ‘p38 mitogen-activated protein kinase-dependent and independent pathways leading to apoptosis in human neutrophils.’ *Journal of Biological Chemistry* 273, 8389-8397.

Fryer RM, Pratt PF, Hsu AK, Gross DJ (2001) ‘Differential activation of extracellular signal regulated kinase isoforms in preconditioning and opioid-induced cardioprotection.’ *Journal of Pharmacology and Experimental Therapeutics* 296, 642-649.

Fujio Y, Nguyen T, Wencker D, Kitsis RN, Walsh K (2000) 'Akt promotes survival of cardiomyocytes in vitro and protects against ischemia–reperfusion injury in mouse heart.' *Circulation* 101, (6) 660-667.

Gao WD, Atar D, Liu Y, Perez NG, Murphy AM, Marban E (1997) 'Role of troponin I proteolysis in the pathogenesis of stunned myocardium.' *Circulation Research* 80, 393-399.

Gao WD, Liu Y, Mellgren R, Marban E (1996) 'Intrinsic myofilament alterations underlying the decreased contractility of stunned myocardium: a consequence of Ca2+-dependent proteolysis?' *Circulation Research* 78, 455-465.

Gavrieli Y, Sherman Y, Ben-Sasson SA (1992) 'Identification of programmed cell death in situ via specific labelling of nuclear DNA fragmentation.' *The Journal of Cell Biology* 119, (3) 493-501.

Geneva, World Health Organization (Global Programme on Evidence for Health Policy
Gill C, Mestril R, Samali A (2002) 'Losing heart: the role of apoptosis in heart disease a novel therapeutic target?' *FASEB* 16, (2) 135-46.

Goldberg RJ, Currie K, White K, Brieger D, Steg PG, Goodman SG, Dabbous O, Fox KA, Gore JM (2004) 'Six-month outcomes in a multinational registry of patients hospitalized with an acute coronary syndrome (the Global Registry of Acute Coronary Events [GRACE]).' *American Journal of Cardiology* 93, (3) 288-93.

Gomez L, Chavanis N, Argaud L, Chalabreysse L, Gateau-Roesch O, Ninet J, Ovize M (2005) 'Fas-independent mitochondrial damage triggers cardiomyocyte death after ischemia-reperfusion.' *Am J Physiol Heart Circ Physiol* 289,H2153-H2158.

Goto M, Liu Y, Yang XM, Ardell JL, Cohen MV, Downey JM (1995) 'Role of bradykinin in protection of ischemic preconditioning in rabbit hearts.' *Circulation Research* 77, 611-21.

Gottlieb RA, Burleson KO, Kloner RA, Babior BM, Engler RL (1994) 'Reperfusion injury induces apoptosis in rabbit cardiomyocytes.' *The Journal of Clinical Investigation* 94, 1621-1628.

Gottlieb RA, Gruol DL, Zhu JY, Engler RL (1996) 'Preconditioning rabbit cardiomyocytes: role of pH, vacuolar proton ATPase, and apoptosis. *Journal of Clinical Investigation* 97, 2391.

Gottlieb RA, Engler RL (1999) 'Apoptosis in myocardial ischemia reperfusion.' *Ann N Y Acad Sci* 874, 412-426.

Gray J, Haran MM, Schneider K, Vesce S, Ray AM, Owen D, White IR, Cutler P, Davis JB (2001) 'Evidence that inhibition of cathepsin-B contributes to the neuroprotective properties of caspase inhibitor Tyr-Val-Ala-Asp-chloromethyl ketone.' *Journal of Bological Chemistry* 276, (35) 32750-327555.

Green D, Kroemer G (1998) 'The central executioners of apoptosis: caspase or mitochondria?.' *Trends Cell Biol* **8**, 267-271.

Grethe S, Ares MP, Andersson T, Porn-Ares MI (2004) 'p38 MAPK mediates TNF-induced apoptosis in endothelial cells via phosphorylation and downregulation of Bcl-x(L).' *Experimental Cell Research* 298, (2) 632-42.

Griffiths EJ, Halestrap AP (1993) 'Protection by Cyclosporin A of ischemia reperfusion-induced damage in isolated rat hearts.' *Journal of Molecular and Cellular Cardiology* 25, 1461-1469.

Grinwald PM (1982) ‘Calcium uptake during postischemic reperfusion in the isolated rat heart: influence of extracellular sodium.’ *Journal of molecular and cellular cardiology* 14, 359-365.

Gross A, Jockel J, Wei MC, Korsmeyer SJ (1998) ‘Enforced dimerization of BAX results in its translocation, mitochondrial dysfunction and apoptosis.’ *EMBO Journal* 17, (14) 3878-85.

Gross GJ, Auchampach JA (1992) ‘Blockade of ATP-sensitive potassium channels prevents myocardial preconditioning in dogs.’ *Circulation Research* 70, 223-33.

Guicciardi ME, Deussing J, Miyoshi H, Bronk SF, Syingen PA, Peters C, Kaufmann SH, Gores GJ (2000) ‘Cathepsin B contributes to TNF-α-mediated hepatocyte apoptosis by promoting mitochondrial release of cytochrome *c.’ J Clin Invest* 106, (9) 1127-1137.

Guyton AC (1991) *Textbook of medical physiology*. Philadelphia: WB Saunders company 8th edition , 240-3.

Haider N, Kharbanda S, Chandrashekhar Y, et al. (1999) ‘Caspase 3 mediated cleavage of troponin C at evolutionarily conserved calcium binding site: relevance of apoptosis in heart failure.’ *Circulation* 100, I–283.

Halestrap AP, Brennerb C (2003) **‘**The Adenine Nucleotide Translocase: A Central Component of the Mitochondrial Permeability Transition Pore and Key Player in Cell Death.’ *Current Medicinal Chemistry* 10, 1507-1525.

Halestrap AP, Clarke SJ, Javadov SA (2004) ‘Mitochondrial permeability transition pore opening during myocardial reperfusion-a target for cardioprotection.’ C*ardiovascular Research* 61, 372-385.

Halestrap AP, McStay GP, Clarke SJ (2002) 'The permeability transition pore complex: another view.' *Biochimie* 84, 153-166.

Hall PA. (1999) 'Assessing apoptosis: a critical survey' *Endocrine-Related Cancer* 6, 3-8.

Hausenloy DJ, Maddock HL, Baxter GF, Yellon DM (2002) 'Inhibiting mitochondrial permeability transition pore opening: a new paradigm for myocardial preconditioning?' *Cardiovascular Research* 55, 534-543.

Hausenloy DJ, Mocanu MM, Yellon DM (2003) 'Activation of the pro-survival kinase cascades (PI3 kinase–Akt–p70S6K kinase and ERK 1/2–p70S6K kinase) at reperfusion are essential for preconditioning-induced protection.' *Circulation* 108, 1-288 (abstract).

Hausenloy DJ, Yellon DM, Mani-Babu S, Duchen MR (2004) 'Preconditioning protects by inhibiting the mitochondrial permeability transition.' *American Journal of Physiology Heart and Circulatory Physiology* 287, H841-H849.

Hausenloy DJ, Tsang A, Mocanu MM, Yellon DM (2005) 'Ischemic preconditioning protects by activating prosurvival kinases at reperfusion.' Am J Physiol 288, H971-H976.

Hearse DJ (1990) 'Ischemia, reperfusion, and the determinants of tissue injury.' *Cardiovascular Drugs and Therapy* 4, (Suppl. 4) 767-776.

Hearse D, Sutherland F (2000) 'Experimental models for the study of cardiovascular function and disease.' *Pharmacological Research* 41, (6) 597-603.

Hoarse DJ, Bolli R (1992) 'Reperfusion induced injury: manifestations, mechanisms and clinical relevance [review].' *Cardiovascular research* 26, 101-108.

Hoffmann JW, Gilbert TB, Poston RS, Silldorff EP (2004) 'Myocardial reperfusion injury: etiology, mechanisms and therapies.' *The Journal of the American Society of Extra-Corporeal Technology* 36, 391-411.

Hoglen NC, Chen LS, Fisher CD, Hirakawa BP, Groessl T, Contreras PC (2004) 'Characterization of IDN-6556 (3-[2-(2-tert-butylphenylaminooxalyl)-amino]-propionylamino]-4-oxo-5-(2,3,5,6-tetrafluoro-phenoxy)-pentanoic acid): A liver-targeted caspase inhibitor.' *J Pharmacol Exp Ther* 309, 634-640.

Holly TA, Drincic A, Byun Y, Nakamura S, Harris K, Klocke FJ, Cryns VL (1999) 'Caspase inhibition reduces myocyte cell death induced by myocardial ischemia and reperfusion in vivo.' *Journal of Molecular and Cellular Cardiology* 31, (9) 1709-15.

Horvitz HR (1999) 'Genetic control of programmed cell death in the nematode Caenorhabditis elegans.' *Cancer Research* 59, 1701s–1706s.

Hsu H, Shu HB. Pan MG. Goeddel DV (1999) 'TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNF receptor 1 signal transduction pathway?' *Cell* 84, 99-308.

Hu K, Nattel S (1995) 'Mechanisms of ischemic preconditioning in rat hearts. Involvement of alpha 1B-adrenoceptors, pertussis toxin-sensitive G proteins, and protein kinase C.' *Circulation* 92, 2259-65.

Hunter DR, Haworth RA (1979) 'The Ca2+-induced membrane transition in mitochondria. I. The protective mechanisms.' *Archeives of Biochemistry and Biophysics* 195, (2) 453-9.

Hussain A, AL-Rajaibi H, Maddock H (2006) 'Post ischemic administration of A_3 adenosine receptor agonist ameliorates myocardial ischemia reperfusion injury via PI3-Kinase.' *Heart* 92, 4.

Iwamoto H, Miura T, Okamura T, Shirakawa K, Iwatate M, Kawamura S, Tatsuno H, Ikeda Y, Matsuzaki M (1999) 'Calpain inhibitor-1 reduces infarct size and DNA fragmentation of

myocardium in ischemic/reperfused rat heart.' *Journal of Cardiovascular Pharmacology* 33, (4) 580-586.

James RS, Young IS, Cox VM, Goldspink DF, Altringham JD (1996) 'Isometric and isotonic muscle properties as determinants of work loop muscle power output.' *Eur. J. Physiol. (Pflügers Arch.)* 432, (5) 767-774.

Jennings RB, Steenbergen CJ, Reimer KA (1995) 'Myocardial ischemia and reperfusion.' *Monographs in Pathology* 37, 47-80.

Jeremias, I, Kupatt C, Martin-Villalba A, Habazettl H, Schenkel J, Boekstegers P, Debatin KM (2000) 'Involvement of CD95/Apo1/Fas in cell death after myocardial ischemia.' *Circulation* 102, 915-920.

Jimenez B, Volpert V, Crawford SE, Febbraio M, Silverstein RL, Bouck N (2000) 'Signals leading to apoptosis-dependent inhibition of neovascularization by thrombospondin-1.' *Nat. Med.* 6, 41–48.

Jonassen AK, Sack MN, Mjos OD, Yellon DM (2001) 'Myocardial protection by insulin at reperfusion requires early administration and is mediated via Akt and p70s6 kinase cell-survival signaling.' *Circulation Research* 89, (12) 1191-1198.

Jordan JE, Zhao ZQ, Vinten-Johansen J (1999) 'The role of neutrophils in myocardial ischemia-reperfusion injury.' *Cardiovasular Research* 43, 860-878.

Josephson RK (1985) 'Mechanical power output from striated muscle during cyclic contraction.' *J. exp. Biol*. 114, 493-512.

Kajstura J, Cheng W, Reiss K, Clark WA, Sonnenblick EH, Krajewski S, Reed JC, Olivetti G, Anversa P (1996) 'Apoptotic and necrotic myocyte cell deaths are independent contributing variables of infarct size in rats.' *Laboratory Investigation* 74, (1) 86-107.

Kang PM, Haunstetter A, Aoki H, Usheva A, Izumo S (2000) 'Morphological and molecular characterization of adlt cardiomyocytes apoptosis during hypoxia and reoxygenation.' *Cardiovascular Research* 87, 118.

Kanoh M, Takemura G, Misao J, Hayakawa Y, Aoyama T, Nishigaki K, Noda T, Fujiwara T, Fukuda K, Minatoguchi S, Fujiwara H (1999) 'Significance of myocytes with positive DNA in situ end-labeling (TUNEL) in hearts with dilated cardiomyopathy: not apoptosis but DNA repair.' *Circulation* 99, (21) 2757-2764.

Kennedy SG, Kandel ES, Cross TK, Hay N (1999) 'Akt/Protein kinase B inhibits cell death by preventing the release of cytochrome *c* from mitochondria.' *Molecular and Cellular Biology* 19, (8) 5800-5810.

Kerr J. F., Wyllie A. H., Currie A. R (1972) 'Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics.' *British Journal of Cancer* 26, 239-257.

Kevin LG, Novalija E, Stowe DF (2005) 'Reactive oxygen species as mediators of cardiac injury and protection: the relevance of anaesthesia practice.' *Anesthesia & Analgesia* 101, 1275-87.

Kevin LG, Novalija.E, Stowe DF (2005) 'Reactive oxygen species as mediators of cardiac injury and protection: the relevance of anaesthesia practice.' *Anesthesia & Analgesia* 101, 1275-87.

Khalil PN, Neuhof C, Huss R, Pollhammer M, Khalil MN (2005) 'Calpain inhibition reduces infarct size and improves global hemodynamics and left ventricular contractility in

a porcine myocardial ischemia/reperfusion model.' *European Journal of Pharmacology* 528, (1-3) 124-31.

Kirshenbaum LA, de Moissac D (1997) 'The bcl-2 gene product prevents programmed cell death of ventricular myocytes.' *Circulation* 96, 1580-1585.

Kloner RA, Jennings RB (2001) 'Consequences of brief ischemia: stunning, preconditioning, and their clinical implications: part I.' *Circulation* 104, 2981-2989.

Kloner RA, Speakman MT, Przyklenk K (2002) 'Ischemic preconditioning: a plea for rationally targeted clinical trials.' *Cardiovascular Research* 55, 526-33.

Kockx MM, Muhring J, Knaapen MW, De Meyer GR (1998) 'RNA synthysis and splicing nterfers with DNA in situ end labeling techniques used to detect apoptosis.' *American Journal of Pathology* 152, (4) 885-8858.

Kohler C, Orrenius S, Zhivotovsky B (2002) 'Evaluation of caspase activity in apoptotic cells.' Journal of Immunological Methods 265, (1-2) 97-110.

Komoriya A, Packard BZ, Brown MJ, Wu M, Henkart PA (2000) 'Assessment of Caspase Activities in Intact Apoptotic Thymocytes Using Cell-permeable Fluorogenic Caspase Substrates.' J. Exp. Med 191, 1819-1828.

Krebs H A & Henseleit K. (1932) 'Studies on urea formation in the animal organism. *Zeitschrift fur Physikalische Chemie* 210, 33-66.

Kroemer G, Dallaporta B, Resche-Rigon M (1998) 'The mitochondrial death/life regulator in apoptosis and necrosis.' *Annual Reviews Physiology* 60, 619-42.

Krown K, Yasui K, Brooker M, Dubin A, Nguyen C, Harris G, McDonough P,. Glembotski C, Palade P, Sabbadini R (1995) 'TNFα receptor expression in rat cardiac myocytes: TNFα inhibition of L-type Ca^{2+} current and Ca^{2+} transients.' *FEBS Letters* 376, 24-30.

Langendorff O (1895) 'Langendorff, Untersuchungen am überlebenden Säugethierherzen.' *Pflügers Arch.* 61, 291-332.

Layland J, Yong I, John D (1997) 'The effects of adrenaline on the work-and power-generating capacity of rat papillary muscle in vitro.' *The journal of experimental biology* 200, 503-509.

Lazebnik YA, Kaufmann SH, Desnoyers S, Poirier GG, Earnshaw WC (1994) 'Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE.' *Nature (London)* 371, 346-347.

Leist M, Single B, Castoldi AF, Kuhnle S, Nicotera P (1997) 'Intracellular adenosine triphosphate (ATP) concentration: a switch in the decision between apoptosis and necrosis.' *Journal of Experimental Medicine* 185,(8) 1481-1486.

Le-Niculescu H, Bonfoco E, Kasuva Y, Claret FX, Green DR, Karin M (1999) 'Withdrawal of survival factors results in activation of the JNK pathway in neuronal cells leading to Fas ligand induction and cell death.' *Molecular and Cellular Biology* 19, 751-763.

Levine B, Kalman J, Mayer L, Fillit H, Packer M (1990) 'Elevated circulating levels of tumor necrosis factor in severe chronic heart failure.' *New England Journal of Medicine*.323, 236-241.

Levitsky S, Feinberg H (1975) 'Biochemical changes of ischemia.' *Annals of Thoracic Surgery* 20, 21-29.

Li H, Zhu H, Xu CJ, Yuan J (1998) 'Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis.' *Cell* 94, 491-501.

Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, Wang X (1997) 'Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade.' *Cell* 91, (4) 479-489.

Li Y, Sato T (2001) 'Dual signaling via protein kinase C and phosphatidylinositol 3-kinase/Akt contributes to bradykinin B2 receptor-induced cardioprotection in guinea pig hearts.' *Journal of Molecular and Cellular Cardiology* 33, (11) 2047-2053.

Liu GS, Thornton J, Winkle DM van, Stanley AW, Olsson RA, Downey JM (1991) 'Protection against infarction afforded by preconditioning is mediated by A1 adenosine receptors in rabbit heart.' *Circulation* 84, 350-6.

Liu X, Kim CN, Yang J, Jemmerson R, Wang X (1996) 'Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c.' *Cell* 86, (1) 147-157.

Loo DT, Rillema JR (1998) 'Measurement of cell death.' *Cell Biology* 57, 251-264.

Lopez AD, Murray CCJL (1998) 'The global burden of disease, 1990–2020.' *Nature Medicine* 4, 1241-1243.

Lucchesi BR (1997) 'Lethal myocardial reperfusion injury.' *Journal of Thrombosis and Thrombolysis* 4, 55-57.

Lundberg K, Szweda LI (2004) 'Initiation of mitochondrial-mediated apoptosis during cardiac reperfusion.' *Archives of Biochemistry and Biophysics* 432, 50-57.

Mandic A, Hansson J, Linder S, Shoshan MC (2003) 'Cisplatin induces ER stress and nucleus-independent apoptotic signaling.' *Journal of Biological Chemistry* 278, 9100-9106.

Maddock.H.L., Mocanu.M.M., Yellon.D.M (2003) 'Adenosine A_3 receptor activation protects the myocardium from reperfusion/reoxygenation injury.' *Am J Heart Circ Physiol*. 283, H1307-1313.

Martin SJ, Reutelingsperger CPM, McGahon AJ, Rader JA, Vanschie RCA, Laface DM & Green DR (1995) 'Early redistribution of plasma-membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus - inhibition by overexpression of Bcl-2 and Abl.' *Journal of Experimental Medicine* 182, 1545-1556.

Mathers CD, Stein C, Ma Fat D, Rao C, Inoue M, Tomijima N, Berbard C, Lopez AD, Murray CJL (2002) *Global Burden of Disease 2000: version 2 methods and results*. Geneva, World Health Organization (Global Programme on Evidence for Health Policy Discussion Paper No. 50). (Internet communication at web site http://www.who.int/evidence/bod).

Matsui T, Li L, del M, Fuku Yi, Franke TF, Hajjar RJ, et al. (1999) 'Adenoviral gene transfer of activated phosphatidylinositol 3'-kinase and Akt inhibits apoptosis of hypoxic cardiomyocytes in vitro.' *Circulation* 100, (23) 2373-2379.

Maulik N, Engelman RM, Rousou JA, Flack JE, 3rd, Deaton D, Das DK (1999) 'Ischemic preconditioning reduces apoptosis by upregulating anti-death gene Bcl-2.' *Circulation* 100, (Suppl.) II369-II375

Maulik N, Yoshida T, Das DK (1998) 'Oxidative stress developed during the reperfusion of ischemic myocardium induces apoptosis.' *Free Radical Biology & Medicine* 24, 869-75.

Maxwell S.R, Lip GY (1997) 'Reperfusion injury: a review of the pathophysiology, clinical manifestations and therapeutic options.' *International Journal of Cardiology* 58, (2) 95-117.

Mayo LD, Donner DB (2001) ‘A phosphatidylinositol 3-kinase/Akt pathway promotes translocation of Mdm2 from the cytoplasm to the nucleus.’ *Proc. Natl. Acad. Sci. U. S. A.* 98, (20) 11598-11603.

McCully JD, Wakiyama H, Hsieh Y, Jones M, Levitsky S (2004) **‘**Differential contribution of necrosis and apoptosis in myocardial ischemia-reperfusion injury.’ *American Journal of Physiology Heart and Circulatory Physiology* 286, H1923-H1935.

McGinnis KM, Gnegy ME, PARK YH, Mukerjee N, Wang KK (1999) ‘Procaspase-3 and poly(ADP)ribose poly- merase (PARP) are calpain substrates.’ *Biochem Biophys Res Commun* 263, 94-99.

Mehta JL, Nichols WW, Mehta P (1988) ‘Neutrophils as potential participants in acute myocardial ischemia: relevance to reperfusion.’ *American college of cardiology* 11, (6) 1309-1316.

Merante, F, Mickle DA, Weisel RD, Li RK, Tumiati LC, Rao V, et al. (1998) ‘Myocardial aerobic metabolism is impaired in a cell culture model of cyanotic heart disease.’ *American Journal f Physiology* 275, (5 Pt 2) H1673-H1681.

Mocanu MM, Baxter GF, Yellon DM (2000) ‘Caspase inhibition and limitation of myocardial infarct size: protection against lethal reperfusion injury.’ *British Journal of Pharmacology* 130, (2) 197-200.

Mort J, Buttle D (1997) ‘Cathepsin B.’ *International Journal of Biochemistry & Cellular Biology* 29, 715-720.

Mukae N, Enari M, Sakahira H, Fukuda Y, Inazawa J, Toh H, Nagata S (1998) ‘Molecular cloning and characterization of human caspase-activated DNase.’ *Proc Natl Acad Sci USA* 95, 9123-9128.

Murray CJL (2002) *Global Burden of Disease 2000: version 2 methods and results*.

Murry CE, Jennings DD, Rios C, Heistad DD, and Davidson B (1986) 'Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium.' *Circulation* 74, 1124-1136.

Muzio M, Chinnaiyan AM, Kischkel FC, O'Rourke K, Shevchenko A, Ni J, Scaffidi C, Bretz JD, Zhang M, Gentz R, Mann M, Krammer PH, Peter ME, and Dixit VM (1996) 'FLICE, a novel FADD-homologous ICE/CED-3 like-protease, is recruited to the CD95 /Fas/Apo-I) death inducing signaling complex.' *Cell* 85, 817-827.

Nakagami H, Morishita R, Yamamoto K, Yoshimura S, Taniyama Y, Aoki M, Matsubara H, Kim S, Kaneda Y, Ogihara T (2001) 'Phosphorylation of p38 mitogen-activated protein kinase downstream of bax-caspase-3 pathway leads to cell death induced by high D-glucose in human endothelial cells.' *Diabetes* 50, 1472–1481.

Nakagawa T, Yuan JY (2000) 'Cross-talk between two cysteine protease families: Activation of caspase-12 by calpain in apoptosis.' *Journal of Cell Biology* 150, 887-894.

Nakamura M, Wang NP, Zhao ZQ, Wilcox JN, Thourani VH, Guyton RA, Vinten-Johansen J (2000) 'Preconditioning decreases Bax expression, PMN accumulation and apoptosis in reperfused rat heart.' *Cardiovascular Research* 45, 661-670.

Narula J, Haider N, Virmani R, et al. (1996) 'Apoptosis in myocytes in end-stage heart failure.' *N Engl J Med* 335, 1182-1189.

Nazareth W, Yafei N, Crompton M (1991) 'Inhibition of anoxia-induced injury in heart myocytes by Cyclosporin-A.' *Journal of Molecular and Cellular Cardiology* 23, 1351-1354.

Nicholson DV, Ali A, Thornberry NA, Vaillancourt JP, Ding CK, Gallant M, Gareau Y, Griffin PR, Labelle M, Lazebnik YA, Munday NA, Raju SM, Smulson ME, Yamin TT, Yu VL, Miller DK (1995) ‘ Identification and inhibition of the ICE/CED-3 protease necessary for mam malian apoptosis.’ *Nature* 376, 37.

Nicholson DW (1999) ‘Caspase structure, proteolytic substrates, and function during apoptotic cell death.’ *Cell Death and Differentiation* 6, 1028-1042.

Ogasawara, J, Watanabe-Fukunaga R, Adachi M, Matsuzawa A, Kasugai T, Kitamura Y, Itoh N, Suda T, Nagata S (1993) ‘Lethal effect of the anti-Fas antibody in mice.’ *Nature* 364, 806-809.

Okamura T, Miura T, Takemura G, Fujiwara H, Iwamoto H, Kawamura S, Kimura M, Ikeda Y, Iwatate M, Matsuzaki M (2000) ‘Effect of caspase inhibitors on myocardial infarct size and myocyte DNA fragmentation in the ischemia-reperfused rat heart.’ *Cardiovascular Research* 45, (3) 642-650.

Okrainec k, Banerjee DK, Eisenberg MJ (2004) ‘Coronary artery disease.’ American Heart Journal 148, (1) 7-15.

Olivetti G, Quaini F, Sala R, Lagrasta C, Corradi D, Bonacina E, Gambert SR, Cigola E, Anversa P (1996) ‘Acute myocardial infarction in humans is associated with activation of programmed myocyte cell death in the surviving portion of the heart.’ *Journal of Molecular and Cellular Cardiology* 28, (9) 2005-2016.

Park JL, Lucchesi BR (1999) ‘Mechanisms of myocardial reperfusion injury.’ *Annals of Thoracic Surgery* 68, (5) 1905-1912.

Parrizas M, Saltiel AR, LeRoith D (1997) 'Insulin-like growth factor 1 inhibits apoptosis using the phosphatidylinositol 3-kinase and mitogen-activated protein kinase pathways.' *Journal of Biological Chemistry* 272, (1) 154-161.

Pasternak R, Braunwald E, Sobel B, Braunwald E (1988) 'Acute myocardial infarction. In: A Textbook of Cardiovascular Medicine.' 3rd edn. Philadelphia: Saunders Company, 1222–1313.

Patterson CE, Rhoades RA (1988) 'Protective role of sulfhydryl reagents in oxidant lung injury.' *Experimental Lung Research* 14, (Suppl) 1005-1019.

Perrin C, Ecarnot-Laubriet, Vergely C, Rochette L (2003) 'Calpain and caspase-3 inhibitors reduce infarct size and post-ischemic apoptosis in rat heart without modifying contractile recovery.' *Cell Mol Biol (Noisy-le-grand).*49, OL497-505.

Przyklenk K (2001) 'Pharmacologic treatment of the stunned myocardium: the concepts and the challenges.' *Coronary Artery Disease* 12, 363-369.

Rabkin SW, Kong JY (2000) 'Nifedipine does not induce but rather prevents apoptosis in cardiomyocytes.' *European Journal of Pharmacology* 388, 209-217.

Rao V, Borger MA, Weisel RD, Ivanov J, Borger MA, Cohen G (2000) 'Insulin cardioplegia for elective coronary bypass surgery.' *Journal of Thoracic and Cardiovascular Surgery* 119, 1176-1184.

Rao, V., Ivanov J, Weisel RD, et al. (2001) 'Lactate release during reperfusion predicts low cardiac output syndrome after coronary bypass surgery.' *Annals of Thoracic Surgery* 71, (6) 1925-30.

Rawles JM (1997) ‘Quantification of the benefit of earlier thrombolytic therapy: five-year results of the Grampian Region Early Anistreplase Trial (GREAT).’ *Journal of American College of Cardiology* 30, (5) 1181-1186.

Razandi M, Pedram A, Levin ER (2000) ‘Estrogen signals to the preservation of endothelial cell form and function.’ *J. Biol. Chem.* 275, 38540–38546.

Reimer KA, Jennings RB (1986) ‘Myocardial ischemia, hypoxia and infarction.’ In: Fozzard HA, Harber E, Jennings RB, Katz AM, Morgan HE eds. *The heart and cardiovascular system.* New York, NY: Raven Press Publishers.

Romashkova JA, Makarov SS (1999) ‘NF-kappaB is a target of AKT in anti-apoptotic PDGF signalling.’ *Nature* 401, (6748) 86-90.

Romson L, Hook BG, Kunkel SL, Abrams GD, Schork MA, Lucchesi BR (1983) ‘Reduction of the extent of ischemic myocardial injury by neutrophil depletion in the dog.’ *Circulation* 67, 1016-1023.

Ross.R (1999) ‘Atherosclerosis: an inflammatory disease.’ *New England Journal of Medicine* 340,115-126.

Ruchaud S, Korfali1 N,Villa1 P, Kottke T, Dingwall C,Kaufmann S,Earnshaw W (2002) ‘Caspase-6 gene disruption reveals a requirement for lamin A cleavage in apoptotic chromatin condensation.’ *EMBO Journal* 21, 1967-1977.

Ruetten H, Badorff C, Ihling C, Zeiher AM, Dimmeler S (2001) ‘Inhibition of caspase-3 improves contractile recovery of stunned myocardium, independent of apoptosis-inhibitory effects.’ *Journal of the American College of Cardiology* 38, (7) 2063-2070.

Sakahira H, Enari M, Nagata S (1998) ‘Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis.’ *Nature* 391, 96-99.

Saraste A, Pulkki K, Kallajoki M, Henriksen K, Parvinen M, Voipio-Pulkki LM (1997) ‘Apoptosis in human acute myocardial infarction.’ *Circulation* 95**,** 320-323.

Schaper W, Schaper J (1997) ‘Reperfusion injury: an opinionated view.’*Journal of Thrombosis and Thrombolysis* 4, 113-116.

Scarabelli TM, Stephanou A, Pasini E, Comini L, Raddino R, Knight RA, Latchman DS (2002) ‘Different Signaling Pathways Induce Apoptosis in Endothelial Cells and Cardiac Myocytes During Ischemia/Reperfusion Injury.’ *Circulation Research* 90, 745-748.

Scarabelli TM, Stephanou A, Pasini E, Gitti G, Townsend P, Lawrence K, Chen-Scarabelli C, Saravolatz L, Latchman D, Knight R, Gardin J (2004) ‘Minocycline inhibits caspase activation and reactivation, increases the ratio of XIAP to smac/DIABLO, and reduces the mitochondrial leakage of cytochrome C and smac/DIABLO.’ *J Am Coll Cardiol* 43, 865-874.

Schotte P, Van Criekinge W, Van de Craen M, Van Loo G, Desmedt M, Grooten,J, Cornelissen M, De Ridder L, Vandekerckhove J, Fiers W, Vandenabeele P, Beyaert R (1998) ‘Cathepsin B-Mediated Activation of the Proinflammatory Caspase-11.’*Biochem. Biophys Res Commun* 251, (1) 379-387.

Schulman D, Latchman DS, Yellon DM (2002) ‘Urocortin protects the heart from reperfusion injury via upregulation of p42/p44 MAPK signaling pathway.’ *American Journal of Physiology Heart and Circulatory Physiology* 283, (4) H1481–H1488.

Schulte G, Fredholm BB (2000) ‘Human adenosine A(1), A(2A), A(2B), and A(3) receptors expressed in Chinese hamster ovary cells all mediate the phosphorylation of extracellular-regulated kinase 1/2.’ *Molecular Pharmacology* 58, (3) 477-482.

Schultz JE, Hsu AK, Gross GJ (1998) ‘Ischemic preconditioning in the intact rat heart is mediated by delta1- but not mu- or kappa-opioid receptors.’ *Circulation* 97, 1282-1289.

Searle J, Kerr J F, Bishop C J (1982) ‘Necrosis and apoptosis: distinct modes of cell death with fundamentally different significance.’ *Pathol. Ann.* 17, 229-259.

Seger R, Krebs EG (1995) ‘The MAPK signaling cascade.’ *FASEB* 9, 726–735.

Semafuko W, Bowie WC (1975) ‘Papillary muscle dynamics: *in situ* function and responses of the papillary muscle.’ *American Journal of Physiology* 228, 1800-1807.

Shimizu N, Yoshiyama M, Omura T, Hanatani A, Kim S, Takeuchi K, et al. (1998)‘Activation of mitogen-activated protein kinases and activator protein-1 in myocardial infarction in rats.’ *Cardiovascular Research* 38, (1) 116-124.

Shimizu S, Eguchi Y, Kamiike W, Waguri S, Uchiyama Y, Matsuda H, Tsujimoto Y (1996 a) ‘Bcl-2 expression prevents activation of the ICE protease cascade.’ *Oncogene.* 12, 2251-2257.

Shimizu S, Eguchi Y, Kamiike W, Waguri S, Uchiyama Y, Matsuda H, Tsujimoto Y (1996 b) ‘Bcl-2 blocks loss of mitochondrial membrane potential while ICE inhibitors act at a different step during inhibition of death induced by respiratory chain inhibitors.’ *Oncogene*. 13, 21-29.

Shimizu S, Egucho Y, Kamiike W, Waguri S, Uchiyama Y, Matsuda H, Tsjimoto Y (1996 c) ‘Retardation of chemical hypoxia-induced necrotic cell death by Bcl-2 and ICE

inhibitors: possible involvement of common mediators in apoptotic and necrotic signal transductions.' *Oncogene* 12, (10) 2045-50.

Shimizu G, Wiegner AW, Gaasch WH, Conrad CH, Cicogna AC, Bing OH (1996 d) 'Force pattern of hypoxic myocardium applied to oxygenated muscle preparations: comparison with effects of regional ischemia on the contraction of non-ischemic myocardium.' *Cardiovasc Res.*32,(6)1038-46.

Simpson PJ, Todd RF III, Fantone JC, Gallagher KP, Lee KA, Tamura Y, Cronin M, Lucchesi BR (1990) 'Sustained limitation of myocardial reperfusion injury by a monoclonal antibody that alters leukocyte function.' *Circulation.* 81,226-237.

Singal PK, Beamish RE, Dhalla NS (1983) 'Potential oxidative pathways of catecholamines in the formation of lipid peroxides and genesis of heart disease.' *Advances in Experimental Medicine and Biology* 161, 391-401.

Slee EA, Harte MT, Kluck RM, Wolf BB, Casiano CA, Newmeyer DD, et al. (1999)'Ordering the cytochrome *c*-initiated caspase cascade: hierarchical activation of caspase-2, -3, -6, -7, -8, and -10 in a caspase-9-dependent manner.' *Journal of Cell Biology* 144, 281-292.

Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, et al. (1985) 'Measurement of protein using bicinchoninic acid.' *Anal Biochem* 150,76-85.

Smulson ME, Simbulan-Rosenthal CM, Boulares AH, Yakovlev A, Stoica B, Iyer S, Luo R, Haddad B, Wang ZQ, Pang T, Jung M, Dritschilo A, Rosenthal DS (2000) 'Roles of poly (ADP-ribosyl)ation and PARP in apoptosis, DNA repair, genomic stability and functions of p53 and E2F-1.' *Advances in Enzyme Regulation* 40, 183-215.

Solenkoya NV, Solodushko V, Cohen MV, Downey JM (2006) 'Endogenous adenosine protects preconditioned heart during early minutes of reperfusion by activating Akt.' *American Journal of Physiology Heart and Circulatory physiology* 290, (1) H441-H449.

Stoka V, Turk B, Schendel SL, Kim TH, Cirman T, Snipas SJ, Ellerby LM, Bredesen D, Freeze H, Abrahamson M, Bromme D, Krajewski S, Reed JC, Yin XM, Turk V, Salvesen GS (2001) 'Lysosomal protease pathways to apoptosis. Cleavage of bid, not pro-caspase, is the most likely route.' *J Biol Chem* 276, (5) 3149–3157.

Strickler J, Jacobson KA, Liang BT (1996) 'Direct preconditioning of cultured chick ventricular myocytes. Novel functions of cardiac adenosine A2a and A3 receptors.' Journal of Clinical Investigation 98, (8) 1773-9.

Su B, Karin M (1996) 'Mitogen-activated protein kinase cascades and regulation of gene expression.' *Current Opinion in Immunology* 8, 402-411.

Sutherland F, Hearse D (2000) 'The isolated blood and perfusion fluid perfused heart.' *Pharmacological Reseach* 41, (6) 613-627.

Talmor D, Applebaum A, Rudich A, Shapira Y, Tirosh A (2000) 'Activation of mitogen-activated protein kinases in human heart during cardiopulmonary bypass.' *Circulation Research* 86, (9) 1004-7.

Tanaka M, Ito H, Adachi S, Akimoto H, Nishikawa T, Kasajima T, Marumo F, Hiroe M (1994) 'Hypoxia induces apoptosis with enhanced expression of Fas antigen messenger RNA in cultured neonatal rat cardiomyocytes.' *Circulation Research* 75, 426-433.

Tavazzi B, Di Pierro D, Bartolini M, et al. (1998) 'Lipid peroxidation, tissue necrosis, and metabolic and mechanical recovery of isolated reperfused rat heart as a function of increasing ischemia.' *Free Radical Research* 28, 25-37.

Terada K, Kaziro Y, Satoh T (2000) ‘Analysis of Ras-dependent signals that prevent caspase-3 activation and apoptosis induced by cytokine deprivation in hematopoietic cells.’ *Biochemical and Biophysical Research Communities* 267, (1) 449-455.

Thom T, Haase N, Rosemond W, Howard VJ, et al. (2006) ‘Heart Disease and Stroke Statistics—2006 Update: A Report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee.’ *Circulation* 113, (6) e85-e151.

Thornberry NA, Bull HG, Calaycay JR, Chapman KT, Howard AD, Kostura MJ, Miller DK, Molineaux SM, Weidner JR, Aunins J (1992) ‘A novel heterodimeric cysteine protease is required for interleukin-1β processing in monocytes.’ *Nature (London)* 356, 768-774.

Thornberry NA, Lazebnik Y (1998) ‘Caspase: enemies within.’ *Science* 281, 1312-1316.

Thornberry NA, Rano TA, Peterson EP, Rasper DM, Timkey T, Garcia-Calvo M, Houtzager VM, Nordstrom PA, Roy S, Vaillancourt JP, et al. (1997) ‘A combinatorial approach defines specificities of members of the caspase family and granzyme B Functional relationships established for key mediators of apoptosis.’ *Journal of Biological Chemistry* 272, 17907-17911.

Thourani VH, Nakamura M, Duarte IG, Bufkin BL, Zhao ZQ, Jordan JE, Shearer ST, Guyton RA, and Vinten-Johansen J (1999) ‘Ischemic preconditioning attenuates postischemic coronary artery endothelial dysfunction in a model of minimally invasive direct coronary artery bypass.’ Jo*urnal of Thoracic and Cardiovascular Surgery* 117, 383-389.

Thorpe GH, Kricka LJ (1986) ‘Enhanced Chemiluminescent Reactions Catalyzed by Horseradish Peroxidase.’ *Methods in Enzymology* 133, 331-353.

Tomai F, Crea F, Chiariello L, Gioffre PA (2001) ‘Ischemic preconditioning in humans: models, mediators, and clinical relevance.’ *Circulation* 100, 559-563.

Tong H, Chen W, Steenbergen C, Murphy E (2000) ‘Ischemic preconditioning activates phosphatidylinositol-3-kinase upstream of protein kinase C.’ *Circulation Research* 87, 309-315.

Tsang A, Hausenloy DJ, Mocanu MM, Yellon DM (2004) ‘Postconditioning: a form of “modified reperfusion” protects the myocardium by activating the phosphatidylinositol 3-kinase–Akt pathway.’ *Circulation Research* 95, 230-232.

Tsuchida K, Aihara H, Isogai K, Hanada K, Shibata N (1986) ‘Degradation of myocardial structural proteins in myocardial infarcted dogs is reduced by Ep459, a cysteine proteinase inhibitor.’ *Biol Chem Hoppe Seyler* 367, 39-45.

Uchiyama T, Engelman RM, Maulik N, Das DK. (2004) ‘Role of Akt signalling in mitochondrial survival pathway triggered by hypoxic preconditioning.’ *Circulation* 109, 3042-3049.

Valentino KL, Gutierrez M, Sanchez R, Winship MJ, Shapiro DA (2003) ‘First clinical trial of a novel caspase inhibitor: Anti-apoptotic caspase inhibitor, IDN-6556, improves liver enzymes.’ *Int J Clin Pharmacol Ther* 41, 441–449.

Vander Heiden MG, Chandel NS, Williamson EK, Schumacker PT, Thompson CB (1997) ‘Bcl-xL regulates the membrane potential and volume homeostasis of mitochondria.’ *Cell* 91, (5) 627-37.

Veinot JP, Gattinger DA, Fliss H (1997) ‘Early apoptosis in human myocardial infarcts.’ *Human Pathology* 28, (4) 485-492.

Vercammen D, Beyaert R, Denecker G, et al. (1998) ‘Inhibition of caspase increases the sensitivity of L929 cells to necrosis mediated by tumor necrosis factor.’ *Journal of Experimental Medicine* 187, 1477-1485.

Verhagen AM, Ekert PG, Pakusch M, Silke J, Connolly LM, Reid GE, Moritz RL, Simpson RJ, Vaux DL (2000) ‘Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins.’ *Cell* 102, (1) 43-53.

Verma S, Fedak PWM, Weisel RD, Butany J, Rao V, Maitland A, Li R, Dhillon B, Yau TM (2002) ‘Fundamentals of reperfusion injury for the clinical cardiologist.’ *Circulation* 105, 2332-2336.

Vermes I, Haanen C, Steffens-Nakken H, Reutelingsperger C (1995) ‘A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V.’ Journal of Immunological Methods 184, (1) 39-51.

Vermes I, Haanen C, Reutelingsperger C (2000) ‘Flow cytometry of apoptotic cell death.’ Journal of Immunological Methods 243, 167-190.

Vinten-Johansen J, Zhao ZQ, Zatta AJ, Kin H, Halkos ME, Kerendi F (2005) ‘Postconditioning-A new link in nature's armor against myocardial ischemia-reperfusion injury.’ *Basic Research in Cardiology* 4, 295-310.

von Harsdorf R, Li PF, Dietz R (1999) ‘Signaling pathways in reactive oxygen species-induced cardiomyocyte apoptosis.’ *Circulation* 99, 2934-2941.

Walker DM, Walker JM, Pugsley WB, Pattison CW, Yellon DM (1995) 'Preconditioning in isolated superfused human muscle.' *Journal of Molecular and Cellular Cardiology* 27, 1349-57.

Wang HG, Pathan N, Ethell IM, Krajewski S, Yamaguchi Y, Shibasaki F, McKeon F, Bobo T, Franke TF, Reed JC (1999) 'Ca^{2+} induced apoptosis through calcineurin dephosphorylation of BAD.' *Science* 284, 339-343.

Wang J, Chun HJ, Wong W, Spencer DM, Lenardo MJ (2001) 'Caspase-10 is an initiator caspase in death receptor signaling.' *Proc Natl Acad Sci USA* 98, 13884-13888.

Wang KK (2000) 'Calpain and caspase: Can you tell the difference?' *Trends in Neurosciences* 23, (1) 20-26.

Weston CR, Balmanno K, Chalmers C, Hadfield K, Molton SA, Ley R, et al. (2003) 'Activation of ERK1/2 by deltaRaf-1:ER represses Bim expression independently of the JNK or PI3K pathways.' *Oncogene* 22, (9) 1281-1293.

Whitmarsh AJ, Davis RJ (1996) 'Transcription factor AP-1 regulation by mitogen-activated protein kinase signal transduction pathways.' *Journal of Molecular Medicine* 74, (10) 589-607.

Wiegner AW, Allen GJ, Bing OH (1978) 'Weak and strong myocardium in series: implications for segmental dysfunction.' *Am J Physiol.* 235(6) H776-83.

Wong CK, White HD (2004) 'Acute Myocardial Infarction: Fibrinolytic Therapy.' *Current Treatment Options Cardiovascular Medicine* 1, 15-28.

Wood DE, Newcomb EW (2000) 'Cleavage of Bax enhances its cell death function.' *Experimental Cell Research* 256, 375-382.

Wu W, Lee WL, Wu YY, Chen D, Liu TJ (2000) 'Expression of constitutively active phosphotidylinositol 3 kinase inhibits activation of caspase 3 and apoptosis of cardiac muscle cells.' *Journal of Biological Chemistry* 275, (51) 40113-4019.

Wyllie AH (1980) 'Cell death: The significance of apoptosis.' *International. Review of cell and molecular biology* 68, 251-306.

Yamaguchi H, Wang HG (2001) 'The protein kinase PKB/Akt regulates cell survival and apoptosis by inhibiting Bax conformational change.' *Oncogene* 20, (53) 7779-7786.

Yamaguchi S, Yamaoka M, Okuyama M, Nitoube J, Fukui A, Shirakabe M, Shirakawa K, Nakamura N, Tomoike H (1999) 'Elevated circulating levels and cardiac secretion of soluble Fas ligand in patients with congestive heart failure.' *American Journal of Cardiology* 83, 1500-1503.

Yang XM, Philipp S, Downey JM, Cohen MV (2005) 'Postconditioning's protection is not dependent on circulatin blood factors or cells but involves adenosine receptors and requires PI3-kinase and guanylyl cyclase activation.' *Basic Research in Cardiology* 100, 57-63.

Yang XM, Proctor JB, Cui L, Krieg T, Downey JM, Cohen MV (2004) 'Multiple, brief coronary occlusions during early reperfusion protect rabbit hearts by targeting cell signaling pathways.' *Journal of the American College of Cardiology* 44, 1103-1110.

Yaoita H, Ogawa K, Maehara K, Maruyama Y (1998) 'Attenuation of ischemia/reperfusion injury in rats by a caspase inhibitor.' *Circulation* 97, (3) 276-81.

Yarbrough WM, Mukherjee R, Escobar GP, Sample JA, McLean JE, Dowdy KB, Hendrick JW, Gibson WC, Hardin AE, Mingoia JT, White PC, Stiko A, Armstrong RC, Crawford FA, Spinale FG (2003) 'Pharmacologic inhibition of intracellular caspase after myocardial

infarction attenuates left ventricular remodeling: a potentially novel pathway.' *Journal of Thoracic Cardiovascular surgery* 126, 1892-1899.

Yellon DM, Baxter GF (2000) 'Protecting the ischemic and reperfused myocardium in acute myocardial infarction. Distant dream or near reality?' *Heart* 83, 381-387.

Yellon DM, Downey JM (2003) 'Preconditioning the myocardium: from cellular physiology to clinical cardiology.' *Physiological Reviews* 83, (4) 1113-1151.

Yoshida K, Sorimachi Y, Fujiwara M, Hironaka K (1995) 'Calpain is implicated in rat myocardial injury after ischemia or reperfusion.' *Japanese Circulation Research* 59, (1) 40-8.

Yoshida K, Inui M, Harada K, Saido TC, Sorimachi Y, Ishihara T, Kawashima S, Sobue K (1995) 'Reperfusion of rat heart after brief ischemia produces proteolysis of calspectin by calpain.' *Circ. Res.* 77, 603-610.

Ytrehus YL, Downey JM (1994) 'Preconditioning protects ischemic rabbit heart by protein kinase C activation.' *Am J Physiol Heart Circ Physiol* 266**,** H1145-H1152.

Ytrehus K (2000) 'The ischemic heart-experimental models.' *Pharmacological Research* 42, (3) 193-203.

Yuan J, Shaham S, Ledoux S, Ellis HM, Horvitz HM (1993) 'The C. elegans cell death gene ced-3 encodes a protein similar to mammalian interleukin-1β-converting enzyme.' *Cell* 75, 641-652.

Yue TL, Ohlstein EH, Ruffolo RR (1999) 'Apoptosis: a potential target for discovering novel therapies for cardiovascular diseases.' *Current Opinion in Chemical Biology* 3, (4) 474-480.

Yue TL, Wang C, Gu JL, Ma XL, Kumar S, Lee JC et al. (2000) ‘Inhibition of extracellular signal-regulated kinase enhances ischemia/reoxygenation-induced apoptosis in cultured cardiac myocytes and exaggerates reperfusion injury in isolated perfused heart.’ *Circulation Research* 86, (6) 692-699.

Yue TL, Wang C, Gu JL, Ma XL, Kumar S, Lee JC, Feuerstein GZ, Thomas H, Maleeff B, Ohlstein EH (2000) ‘Inhibition of extracellular signal-regulated kinase enhances Ischemia/Reoxygenation-induced apoptosis in cultured cardiac myocytes and exaggerates reperfusion injury in isolated perfused heart.’ *Circulation Research* 86, (6) 692-699.

Zamzami N, Susin SA, Marchetti P, Hirsch T, Gomez-Monterrey I, Castedo M, Kroemer G (1996) ‘Mitochondrial control of nuclear apoptosis.’ *Journal of Experimental Medicine* 183, (4) 1533-1544.

Zweier JL (1988) ‘Measurement of superoxide derived free radicals in the reperfused heart: evidence for a free radical mechanism of reperfusion injury.’ *Journal of Biological Chemistry* 263, 1353-1357.

Zha J, Harada H, Yang E, Jockel J, Korsmeyer SJ (1996) ‘Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X(L).’ *Cell* 87, (4) 619–628.

Zhao ZQ, Corvera JS, Halkos ME, et al. (2003) ‘Inhibition of myocardial injury by ischemic postconditioning during reperfusion: comparison with preconditioning.’ *American Journal of Heart and Circulation physiology* 285, H579-H588.

Zhao ZQ, Morris CD, Budde JM et al. (2001) ‘Inhibition of apoptosis with aurintricarboxylic acid reduces extension of infarction and improves regional contractile dysfunction during reperfusion.’ *Circulation* 104, (Suppl II) II11.

Zhao ZQ, Morris CD, Budde JM, Wang NP, Muraki S, Sun HY, Guyton RA (2003) ‘Inhibition of myocardial apoptosis reduces infarct size and improves regional contractile dysfunction during reperfusion**.’** *Cardiovascular Research* 59, (1) 132-142.

Zhao ZQ, Nakamura M, Wang NP, Wilcox JN, Shearer S, Ronson RS, Guyton RA, Vinten-Johansen J (2000) ‘Reperfusion induces myocardial apoptotic cell death.’ *Cardiovascular Research* 45, (3) 651-60.

Appendix

Materials

Langendorff Perfusion Studies

Krebs-Henseleit bicarbonate buffer (in mM):
NaCl - 118.5,
$NaHCO_3$ - 25.0
KCl - 4.8
$MgSO_4$ - 1.2
KH_2PO_4 - 1.2
Glucose - 12
$CaCl_2$ - 1.7
Prepared freshly each day prior to each experiment, dissolved and gassed with 95% O_2 / 5% CO_2, at a pH of 7.4-7.5 at 37 °C.

Evans blue (Sigma):
0.25 % w/v Evans blue in 100ml 10% saline solution

TTC preparation:
2 mL 0.1M NaH^2PO^4
8 mL 0.1M Na^2HPO^4
1g Triphenyl Tetrazolium Chloride (Sigma)

Western Blotting reagents

Sample Buffer
Tris-HCl 250 mM, pH6.8
Glycerol 10%
Bromophenol Blue 0.006%
SDS 4%
ß-mercaptoethanol 2%, pH 6.8

Protein Lysis Buffer for Akt analysis
NaCl-0.1M
Tris base-10 mM
EDTA (pH 8.0)-1 mM
Sodium pyrophosphate-2mM
NaF-2mM
β-glycerophosphate-2 mM
AEBSF-0.1 mg/ml

Protease cocktail tablet-1/1.5 tablets

Protein lysis buffer for cytochrome c analysis
Mannitol 210 mM
Sucrose 70 mM
EDTA 1mM
PMSF 0.5 mM
Protease inhibitor cocktail 1.5tablet
Cyclosporine 0.1 µM
pH 7.4

Running gel base (100ml)
1.5 M Tris base - 18.16 g
0.4% SDS – 0.4g
DH_2O – 60 ml
Correct to pH 8.8 with hydrochloric acid
make up to100ml with DH_2O

Stacking gel base (100ml)
0.5M Tris base – 6.05 g
0.4% SDS – 0.4 g
DH_2O – 60 ml
make up to 100ml with DH_2O

Running gel (12.5%)
Distilled water – 12 ml
Running gel base – 9 ml
30% acrylamide – 15 ml
TEMED – 40 µl
10% Amonnium persulfate –200 µl

Stacking gel (5%)
Distilled water – 7 ml
Stacking gel base – 3 ml
30% acrylamide – 2 ml
TEMED – 24 µl
10% APS – 120 µl

10% Ammonium Persulfate (APS)
0.1g APS (Sigma)
1000µl ddH_20

10% Sodium Dodecyl Sulfate (SDS)
(solution needs to be pH'd to dissolve)
1g (Sigma)
10ml ddH_20

10X Running buffer
Glycine – 144.2 g
SDS – 10 g
TRIS base – 30.3 g
Distilled water -1 litre
For 1X running buffer diluted 1:10 in ddH_20

10X Transfer buffer
Glycine – 144.2 g
Tris base – 30.3 g
Distilled water – 1 litre

1X Transfer buffer
x10 transfer buffer – 100 ml
20% MeOH – 200 ml
Distilled water – 700 ml

10X TBS/T (Tris Buffered Saline + Tween)
Tris Base – 24.2 g
NACL – 80 g
Correct to pH 7.6 with hydrochloric acid
make up to 1L with DH_2O
Add 1mL Tween (final 0.1%)

Blocking buffer
(5% Milk in TBS/T)
Dehydrated Milk - 1.25 g
TBS/T – 25 ml

Antibody Dilution Buffer
(5% BSA in TBS/T)
Bovine Serum Albumin – 2.5g
TBS/T – 50ml

<u>Isolated Myocyte Studies</u>

Kreb's Henseleit Buffer (in mM):

$Na_2HPO_4.12H_20$ - 0.9
Pyruvate - 5
Taurine - 20
Glucose - 10
$MgSO_4.7.H_20$ - 0.4
KCl - 5.4
NaCl - 116

Kreb's Henseleit Buffer with $CaCl_2$:
200mL Krebs (above)
150ul of 1M $CaCl_2$ Stock

Collagenase Buffer
1g BSA
0.15g Collagenase (Worthington Biochemicals)
200mL Krebs Buffer 2
8.8ul of 1M $CaCl_2$ Stock
pH to 7.4 w/ NaOH

Restoration Buffer
0.131g Creatine
0.064g Carnitine
2g BSA
100ul 100mM $CaCl_2$
2mL Pen-Strep
200mL Krebs Buffer 2

Hypoxic Buffer

	Grams/Liter
12mM KCl	0.89
0.49mM MgCl2	0.09
0.9mM CaCl2	0.13
4mM HEPES	0.953
10mM Deoxyglucose	1.64
20mM Lactate	1.7

<u>Caspase-3 Assay Reagents</u>

6% Formaldehyde
6ml 10% formaldehyde
4ml Phosphate Buffered Saline (PBS)

90% Methanol
45ml 100% HPLC grade MeOH

5ml ddH_20

Incubation Buffer
0.5g BSA
100mL PBS